A process for producing polyunsaturated fatty acids

Description

The present invention relates to a process for producing polyunsaturated fatty acids in an organism by introducing nucleic acids into said organism which code for polypeptides having acyl-CoA:lysophospholipid-acyltransferase activity. Advantageously, these nucleic acid sequences may, if appropriate together with further nucleic acid sequences coding for biosynthesis polypeptides of the fatty acid or lipid metabolism, be expressed in the transgenic organism.

The invention furthermore relates to the nucleic acid sequences, to nucleic acid constructs comprising the nucleic acid sequences of the invention, to vectors comprising said nucleic acid sequences and/or said nucleic acid constructs and to transgenic organisms comprising the abovementioned nucleic acid sequences, nucleic acid constructs and/or vectors.

A further part of the invention relates to oils, lipids and/or fatty acids produced by the process of the invention and to their use.

Fatty acids and triglycerides have a multiplicity of applications in the food industry, in animal nutrition, in cosmetics and in the pharmacological sector. Depending on whether they are free saturated or unsaturated fatty acids or else triglycerides with an elevated content of saturated or unsaturated fatty acids, they are suitable for very different applications; thus, for example, polyunsaturated fatty acids are added to baby food to improve the nutritional value. Polyunsaturated ω-3-fatty acids and ω-6-fatty acids are, in this connection, an important constituent of animal and human food. Owing to the composition of human food, which is customary today, an addition of polyunsaturated ω-3-fatty acids which are preferably present in fish oils to the food is particularly important. Thus, for example, polyunsaturated fatty acids such as docosahexaenoic acid (= DHA, C22:$6^{\Delta 4,7,10,13,16,19}$) or eisosapentaenoic acid (= EPA, C20:$5^{\Delta 5,8,11,14,17}$) are added to baby food to improve the nutritional value. The unsaturated fatty acid DHA is said to have a positive effect on brain development.

Hereinbelow, polyunsaturated fatty acids are referred to as PUFA, PUFAs, LCPUFA or LCPUFAs (**p**oly **u**nsaturated **f**atty **a**cids, **PUFA**, **l**ong **c**hain **p**oly **u**nsaturated **f**atty **a**cids, **LCPUFA**).

The various fatty acids and triglycerides are obtained, usually in the form of their triacylglycerides (= triglycerides = triglycerols), mainly from microorganisms such as Mortierella or Schizochytrium or from oil-producing plants such as soybean, oilseed rape, algae such as Crypthecodinium or Phaeodactylum and others. However, they may also be obtained from animals such as, for example, fish. The free fatty acids are advantageously prepared by hydrolysis. Higher polyunsaturated fatty acids such as DHA, EPA, arachidonic acid (= ARA, C20:$4^{\Delta 5,8,11,14}$), dihomo-γ-linolenic acid (C20:$3^{\Delta 8,11,14}$) or docosapentaenoic acid (DPA, C22:$5^{\Delta 7,10,13,16,19}$) cannot be isolated

from oil crops, such as oilseed rape, soybean, sunflower, safflower or others. Conventional natural sources of these fatty acids are fish such as herring, salmon, sardine, red fish, eel, carp, trout, halibut, mackerel, zander or tuna, or algae.

Depending on the intended application, preference is given to oils with saturated or unsaturated fatty acids; thus, for example, lipids with unsaturated fatty acids, especially polyunsaturated fatty acids, are preferred in human nutrition. The polyunsaturated ω-3-fatty acids are said to have in this connection a positive effect on the cholesterol level in the blood and thus on the possibility of preventing heart disease. The risk of heart disease, stroke or hypertension may be reduced markedly by adding these ω-3-fatty acids to food. ω-3-fatty acids can also have a positive effect on inflammatory, especially chronically inflammatory, processes in connection with immunological disorders such as rheumatoid arthritis. They are therefore added to food, especially dietetic food, or are applied in medicaments. ω-6-fatty acids such as arachidonic acid tend to have a negative effect on these diseases in connection with said rheumatic disorders, due to our customary foodstuff composition.

ω-3- and ω-6-fatty acids are precursors of tissue hormones, the "eicosanoides, such as the prostaglandins, which are derived from dihomo-γ-linolenic acid, arachidonic acid and eicosapentaenoic acid, the thromoxanes and leukotrienes which are derived from arachidonic acid and eicosapentaenoic acid. Eicosanoides ("PG_2 series") which are formed from ω-6-fatty acids normally promote inflammatory reactions, while eicosanoides ("PG_3 series") from ω-3-fatty acids have little or no proinflammatory effect.

Owing to their positive properties, there has been no lack of attempts in the past to make available genes which are involved in the synthesis of fatty acids or triglycerides for the production of oils in various organisms with a modified content of unsaturated fatty acids. Thus, WO 91/13972 and its US equivalent describe a Δ9-desaturase. WO 93/11245 claims a Δ15-desaturase and WO 94/11516 a Δ12-desaturase. Further desaturases are described, for example, in EP-A-0 550 162, WO 94/18337, WO 97/30582, WO 97/21340, WO 95/18222, EP-A-0 794 250, Stukey et al., J. Biol. Chem., 265, 1990: 20144-20149, Wada et al., Nature 347, 1990: 200-203 and Huang et al., Lipids 34, 1999: 649-659. However, the biochemical characterization of the various desaturases has been insufficient to date since the enzymes, being membrane-bound proteins, can be isolated and characterized only with great difficulty (McKeon et al., Methods in Enzymol. 71, 1981: 12141-12147, Wang et al., Plant Physiol. Biochem., 26, 1988: 777-792). Membrane-bound desaturases are normally characterized by being introduced into a suitable organism which is subsequently studied for enzyme activity by analyzing reactants and products. Δ6-desaturases are described in WO 93/06712, US 5,614,393, US 5614393, WO 96/21022, WO 00/21557 and WO 99/27111, as is the application for production in transgenic organisms, namely in WO 98/46763, WO 98/46764, WO 9846765. The expression of various desaturases such as those in WO 99/64616 or WO 98/46776 and the formation of polyunsaturated fatty acids are also described and claimed in this connection. Regarding the efficacy of desaturase

expression and its influence on the formation of polyunsaturated fatty acids, it should be noted that expression of a single desaturase, as described previously, has resulted in only low contents of unsaturated fatty acids/lipids such as, for example, γ-linolenic acid and stearidonic acid. Furthermore, a mixture of ω-3- and ω-6-fatty acids was usually obtained.

Particularly suitable microorganisms for producing PUFAs are microorganisms such as Thraustochytrium or Schizochytrium strains, algae such as Phaeodactylum tricornutum or Crypthecodinium species, ciliates, such as Stylonychia or Colpidium, fungi such as Mortierella, Entomophthora or Mucor. Strain selection has resulted in the development of a number of mutant strains of the corresponding microorganisms, which produce a series of desirable compounds including PUFAs. However, the mutation and selection of strains with improved production of a particular molecule such as the polyunsaturated fatty acids is a time-consuming and difficult process. Therefore, preference is given, whenever possible, to genetic engineering processes, as described above. However, only limited amounts of the desired polyunsaturated fatty acids such as DPA, EPA or ARA can be produced with the aid of the abovementioned microorganisms, and, depending on the microorganism used, the former are usually obtained as fatty acid mixtures of, for example, EPA, DPA and DHA.

Alternatively, fine chemicals may be produced advantageously on a large scale via production in plants which are developed so as to produce the abovementioned PUFAs. Plants which are particularly well suited for this purpose are oil crops which contain large amounts of lipid compounds, such as oilseed rape, canola, linseed, soybean, sunflower, borage and evening primrose. However, other crop plants containing oils or lipids and fatty acids are also well suited, as mentioned in the detailed description of the present invention. Conventional breeding has been used to develop a number of mutant plants which produce a spectrum of desirable lipids and fatty acids, cofactors and enzymes. However, the selection of new plant cultivars with improved production of a particular molecule is a time-consuming and difficult process or even impossible if the compound does not naturally occur in the respective plant, as is the case with polyunsaturated C_{18}-, C_{20}- fatty acids and C_{22}- fatty acids and those having longer carbon chains.

Owing to the positive properties of unsaturated fatty acids, there has been no lack of attempts in the past to make available these genes which are involved in the synthesis of fatty acids or triglycerides for the production of oils in various plants with a modified content of polyunsaturated fatty acids. Previously, however, it was not possible to produce longer-chain polyunsaturated C_{20}- and/or C_{22}- fatty acids such as EPA or ARA in plants.

However, in other organisms as well as microorganisms such as algae or fungi too, genetically engineered modifications of the fatty acid metabolic pathway via introducing and expressing, for example, desaturases resulted only in relatively small increases in productivity in these organisms. One reason for this may be the high complexity of the

fatty acid metabolism. Thus, incorporation of polyunsaturated fatty acids into membrane lipids and/or into triacylglycerides and their degradation and conversion are very complex and, even now, has still not been fully elucidated and understood biochemically and, especially genetically.

The biosynthesis of LCPUFAs and incorporation of LCPUFAs into membranes or triacylglycerides are carried out via various metabolic pathways (Abbadi et al. (2001) European Journal of Lipid Science & Technology 103:106-113). In bacteria such as Vibrio and microalgae such as Schizochytrium, malonyl-CoA is converted via a LCPUFA-producing polyketide synthase to give LCPUFAs (Metz et al. (2001) Science 293: 290-293; WO 00/42195; WO 98/27203; WO 98/55625). In microalgae such as Phaeodactylum and mosses such as Physcomitrella, unsaturated fatty acids such as linoleic acid or linolenic acid are converted in the form of their acyl-CoAs in multiple desaturation and elongation steps to give LCPUFAs (Zank et al. (2000) Biochemical Society Transactions 28: 654-658). In mammals, the biosynthesis of DHA includes β-oxidation, in addition to desaturation and elongation steps.

In microorganisms and lower plants, LCPUFAs are present either exclusively in the form of membrane lipids, as is the case in Physcomitrella and Phaeodactylum, or in membrane lipids and triacylglycerides, as is the case in Schizochytrium and Mortierella. Incorporation of LCPUFAs into lipids and oils is catalyzed by various acyltransferases and transacylases. These enzymes are already known to carry out the incorporation of saturated and unsaturated fatty acids [Slabas (2001) J. Plant Physiology 158: 505-513; Frentzen (1998) Fett/Lipid 100: 161-166); Cases et al. (1998) Proc. Nat. Acad. Sci. USA 95: 13018-13023]. The acyltransferases are enzymes of the "Kennedy pathway", which are located on the cytoplasmic side of the membrane system of the endoplasmic reticulum, referred to as "ER" hereinbelow. ER membranes may be isolated experimentally as "microsomal fractions" from various organisms (Knutzon et al. (1995) Plant Physiology 109: 999-1006; Mishra & Kamisaka (2001) Biochemistry 355: 315-322; US 5968791). These ER-bound acyltransferases in the microsomal fraction use acyl-CoA as the activated form of fatty acids. Glycerol-3-phosphate acyltransferase, referred to as GPAT hereinbelow, catalyzes the incorporation of acyl groups at the sn-1 position of glycerol 3-phosphate. 1-Acylglycerol-3-phosphate acyltransferase (E.C. 2.3.1.51), also known as lysophosphatidic-acid acyltransferase and referred to as LPAAT hereinbelow, catalyzes the incorporation of acyl groups at the sn-2 position of lysophosphatidic acid, abbreviated as LPA hereinbelow. After dephosphorylation of phosphatidic acid by phosphatidic-acid phosphatase, diacylglycerol acyltransferase, referred to as DAGAT hereinbelow, catalyzes the incorporation of acyl groups at the sn-3 position of diacylglycerols. Apart from these Kennedy pathway enzymes, further enzymes capable of incorporating acyl groups from membrane lipids into triacyl-glycerides are involved in the incorporation of fatty acids into triacylglycerides, namely phospholipid diacylglycerol acyltransferase, referred to as PDAT hereinbelow, and lysophosphatidylcholine acyltransferase, referred to as LPCAT.

The enzymic activity of an LPCAT was first described in rats [Land (1960) Journal of

Biological Chemistry 235: 2233-2237]. A plastic LPCAT isoform [Akermoun et al. (2000) Biochemical Society Transactions 28: 713-715] and an ER-bound isoform [Tumaney and Rajasekharan (1999) Biochimica et Biophysica Acta 1439: 47-56; Fraser and Stobart, Biochemical Society Transactions (2000) 28: 715-7718] exist in plants. LPCAT is involved in the biosynthesis and transacylation of polyunsaturated fatty acids in animals as well as in plants [Stymne and Stobart (1984) Biochem. J. 223: 305-314; Stymne und Stobart (1987) in 'The Biochemistry of Plants: a Comprehensive Treatise', Vol. 9 (Stumpf, P.K. ed.) pp. 175-214, Academic Press, New York]. An important function of LPCAT or, more generally, of an acyl-CoA:lysophospholipid acyltransferase, referred to as LPLAT hereinbelow, in the ATP-independent synthesis of acyl-CoA from phospholipids has been described by Yamashita et al. (2001; Journal of Biological Chemistry 276: 26745-26752).

Despite many biochemical data, no genes coding for LPCAT have been identified previously. Genes of various other plant acyltransferases have been isolated and are described in WO 00/18889 (Novel Plant Acyltransferases).

Higher plants comprise polyunsaturated fatty acids such as linoleic acid (C18:2) and linolenic acid (C18:3). Arachidonic acid (ARA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are, as described above, found not at all in the seed oil of higher plants, or only in traces (E. Ucciani: Nouveau Dictionnaire des Huiles Végétales. Technique & Documentation – Lavoisier, 1995. ISBN: 2-7430-0009-0). It is advantageous to produce LCPUFAs in higher plants, preferably in oil seeds such as oilseed rape, linseed, sunflower and soybean, since large amounts of high-quality LCPUFAs for the food industry, animal nutrition and pharmaceutical purposes may be obtained at low costs in this way. To this end, it is advantageous to introduce into and express in oil seeds genes coding for enzymes of the biosynthesis of LCPUFAs by genetic engineering methods. Said genes encode, for example, Δ6-desaturase, Δ6-elongase, Δ5-desaturase, Δ5-elongase and Δ4-desaturase. These genes may advantageously be isolated from microorganisms, animals and lower plants which produce LCPUFAs and incorporate them in the membranes or triacylglycerides. Thus, Δ6-desaturase genes have already been isolated from the moss Physcomitrella patens and Δ6-elongase genes have already been isolated from P. patens and the nematode C. elegans.

First transgenic plants which comprise and express genes coding for enzymes of the LCPUFA biosynthesis and produce LCPUFAs have been described for the first time, for example, in DE 102 19 203 (process for the production of polyunsaturated fatty acids in plants). However, these plants produce LCPUFAs in amounts which require further optimization for processing the oils present in said plants.

In order to enable food and feed to be enriched with these polyunsaturated fatty acids, there is therefore a great need for a simple, inexpensive process for producing said polyunsaturated fatty acids, especially in eukaryotic systems.

It was therefore the object to develop a process for producing polyunsaturated fatty

acids in a eukaryotic organism. This object was achieved by the process according to the invention for producing polyunsaturated fatty acids in an organism, wherein said process comprises the following steps:

a) introducing into the organism at least one nucleic acid sequence having the sequence depicted in SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5 or SEQ ID NO: 7, which sequence codes for a polypeptide having an acyl-CoA:lysophospholipid-acyltransferase activity; or

b) introducing into said organism at least one nucleic acid sequence which can be derived, as a result of the degenerated genetic code, from the coding sequence comprised in SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5 or SEQ ID NO: 7, or

c) introducing into said organism at least one derivative of the nucleic acid sequence depicted in SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5 or SEQ ID NO: 7, which code for polypeptides having the amino acid sequence depicted in SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6 or SEQ ID NO: 8 and which are at least 40% homologous at the amino acid level to SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6 or SEQ ID NO: 8 and have an equivalent acyl-CoA:lysophospholipid-acyltransferase activity, and

d) culturing and harvesting said organism.

Advantageously, the polyunsaturated fatty acids produced in the process of the invention comprise at least two, advantageously three, double bonds. The fatty acids particularly advantageously comprise four or five double bonds. Fatty acids produced in the process advantageously have 16, 18, 20 or 22 carbon atoms in the fatty acid chain. These fatty acids which have been produced may be produced in said process as a single product or be present in a fatty acid mixture.

The nucleic acid sequences used in the process of the invention are isolated nucleic acid sequences which code for polypeptides having acyl-CoA:lysophospholipid-acyl-transferase activity.

The polyunsaturated fatty acids produced in the process are advantageously bound in membrane lipids and/or triacylglycerides but may also occur in the organisms as free fatty acids or else bound in the form of other fatty acid esters. In this context, they may be present as "pure products" or else advantageously in the form of mixtures of various fatty acids or mixtures of different glycerides. The various fatty acids bound in the triacylglycerides can be derived here from short-chain fatty acids having from 4 to 6 carbon atoms, medium-chain fatty acids having from 8 to 12 carbon atoms or long-chain fatty acids having from 14 to 24 carbon atoms, with preference being given to the long-chain fatty acids and particular preference being given to the long-chain fatty acids, LCPUFAs, of C_{18}-, C_{20}- and/or C_{22}- fatty acids.

The process of the invention advantageously produces fatty acid esters with polyunsaturated C_{16}-, C_{18}-, C_{20}- and/or C_{22}-fatty acid molecules, with at least two double bonds being present in the fatty acid ester. These fatty acid molecules preferably comprise three, four or five double bonds and advantageously lead to the synthesis of hexadecadienoic acid (C16:$2^{\Delta 9,12}$), γ-linolenic acid (= GLA, C18:$3^{\Delta 6,9,12}$), stearidonic acid (= SDA, C18:$4^{\Delta 6,9,12,15}$), dihomo-γ-linolenic acid (= DGLA, 20:$3^{\Delta 8,11,14}$), eicosatetraenoic acid (= ETA, C20:$4^{\Delta 5,8,11,14}$), arachidonic acid (ARA), eicosapentaenoic acid (EPA) or mixtures thereof, preferably EPA and/or ARA.

The fatty acid esters with polyunsaturated C_{16}-, C_{18}-, C_{20}- and/or C_{22}-fatty acid molecules can be isolated in the form of an oil or lipid, for example in the form of compounds such as sphingolipids, phosphoglycerides, lipids, glycolipids such as glycosphingolipid, phospholipids such as phosphatidylethanolamine, phosphatidylcholine, phosphatidylserine, phosphatidylglycerol, phosphatidylinositol or diphosphatidylglycerol, monoacylglycerides, diacylglycerides, triacylglycerides or other fatty acid esters such as the acetyl-coenzyme A esters which comprise the polyunsaturated fatty acids with at least two, preferably three double bonds, from the organisms which have been used for the preparation of the fatty acid esters. In addition to these esters, the polyunsaturated fatty acids are also present in the organisms, advantageously the plants as free fatty acids or bound in other compounds. As a rule, the various abovementioned compounds (fatty acid esters and free fatty acids) are present in the organisms with an approximate distribution of 80 to 90% by weight of triglycerides, 2 to 5% by weight of diglycerides, 5 to 10% by weight of monoglycerides, 1 to 5% by weight of free fatty acids, 2 to 8% by weight of phospholipids, the total of the various compounds amounting to 100% by weight.

The process according to the invention yields the LCPUFAs produced in a content of at least 3% by weight, advantageously at least 5% by weight, preferably at least 8% by weight, especially preferably at least 10% by weight, most preferably at least 15% by weight, based on the total fatty acids in the transgenic organisms, preferably in a transgenic plant. Since a plurality of reaction steps are performed by the starting compounds hexadecadienoic acid (C16:2), linoleic acid (C18:2) and linolenic acid (C18:3) in the process according to the invention, the end products of the process such as, for example, arachidonic acid (ARA) or eicosapentaenoic acid (EPA) are not obtained as absolutely pure products; minor traces of the precursors are always present in the end product. If, for example, both linoleic acid and linolenic acid are present in the starting organism and the starting plant, the end products such as ARA and EPA are present as mixtures. The precursors should advantageously not amount to more than 20% by weight, preferably not to more than 15% by weight, especially preferably not to more than 10% by weight, most preferably not to more than 5% by weight, based on the amount of the end product in question. Advantageously, only ARA or only EPA, bound or as free acids, are produced as end products in a transgenic plant owing to the process according to the invention. If both compounds (ARA and EPA) are produced simultaneously, they are advantageously produced in a ratio of at least 1:2 (EPA:ARA), advantageously of at least 1:3, preferably 1:4,

especially preferably 1:5.

Owing to the nucleic acid sequences according to the invention, an increase in the yield of polyunsaturated fatty acids of at least 50%, advantageously of at least 80%, especially advantageously of at least 100%, very especially advantageously of at least 150%, in comparison with the nontransgenic starting organism, can be obtained by comparison in GC analysis (see examples).

Chemically pure polyunsaturated fatty acids or fatty acid compositions can also be synthesized by the processes described above. To this end, the fatty acids or the fatty acid compositions are isolated from the organism, such as the microorganisms or the plants or the culture medium in or on which the organisms have been grown, or from the organism and the culture medium, in the known manner, for example via extraction, distillation, crystallization, chromatography or combinations of these methods. These chemically pure fatty acids or fatty acid compositions are advantageous for applications in the food industry sector, the cosmetics sector and especially the pharmacological industry sector.

Suitable organisms for the production in the process according to the invention are, in principle, any organisms such as fungi, such as Mortierella or Thraustrochytrium, yeasts such as Saccharomyces or Schizosaccharomyces, mosses such as Physcomitrella or Ceratodon, nonhuman animals such as Caenorhabditis, algae such as Crypthecodinium or Phaeodactylum or plants such as dicotyledonous or monocotyledonous plants. Organisms which are especially advantageously used in the process according to the invention are organisms which belong to the oil-producing organisms, that is to say which are used for the production of oils, such as fungi, such as Mortierella or Thraustochytrium, algae such as Crypthecodinium, Phaeodactylum, or plants, in particular plants, preferably oil crop plants which comprise large amounts of lipid compounds, such as peanut, oilseed rape, canola, sunflower, safflower, poppy, mustard, hemp, castor-oil plant, olive, sesame, Calendula, Punica, evening primrose, verbascum, thistle, wild roses, hazelnut, almond, macadamia, avocado, bay, pumpkin/squash, linseed, soybean, pistachios, borage, trees (oil palm, coconut or walnut) or arable crops such as maize, wheat, rye, oats, triticale, rice, barley, cotton, cassava, pepper, Tagetes, Solanaceae plants such as potato, tobacco, eggplant and tomato, Vicia species, pea, alfalfa or bushy plants (coffee, cacao, tea), Salix species, and perennial grasses and fodder crops. Preferred plants according to the invention are oil crop plants such as peanut, oilseed rape, canola, sunflower, safflower, poppy, mustard, hemp, castor-oil plant, olive, Calendula, Punica, evening primrose, pumpkin/squash, linseed, soybean, borage, trees (oil palm, coconut). Especially preferred are plants which are high in C18:2- and/or C18:3-fatty acids, such as sunflower, safflower, tobacco, verbascum, sesame, cotton, pumpkin/squash, poppy, evening primrose, walnut, linseed, hemp, thistle or safflower. Very especially preferred plants are plants such as safflower, sunflower, poppy, evening primrose, walnut, linseed or hemp.

It is advantageous to the inventive process described to introduce, in addition to the nucleic acids introduced in steps (a) to (c) of the process, further nucleic acids which encode enzymes of the fatty acid or lipid metabolism.

In principle, all genes of the fatty acid or lipid metabolism can be used in the process for the production of polyunsaturated fatty acids, advantageously in combination with the inventive acyl-CoA:lysophospholipid acyltransferase. Genes of the fatty acid or lipid metabolism selected from the group consisting of acyl-CoA dehydrogenase(s), acyl-ACP [= acyl carrier protein] desaturase(s), acyl-ACP thioesterase(s), fatty acid acyltransferase(s), fatty acid synthase(s), fatty acid hydroxylase(s), acetyl-coenzyme A carboxylase(s), acyl-coenzyme A oxidase(s), fatty acid desaturase(s), fatty acid acetylenases, lipoxygenases, triacylglycerol lipases, allenoxide synthases, hydroperoxide lyases or fatty acid elongase(s) are advantageously used in combination with the acyl-CoA:lysophospholipid acyltransferase. Genes selected from the group of the Δ4-desaturases, Δ5-desaturases, Δ6-desaturases, Δ8-desaturases, Δ9-desaturases, Δ12-desaturases, Δ5-elongases, Δ6-elongases or Δ9-elongases are especially preferably used in combination with the acyl-CoA:lysophospholipid acyltransferase in the process of the invention.

Owing to the enzymatic activity of the nucleic acids used in the process according to the invention which encode polypeptides with acyl-CoA:lysophospholipid acyltransferase activity, advantageously in combination with nucleic acid sequences which encode polypeptides of the fatty acid or lipid metabolism, such as Δ4-, Δ5-, Δ6-, Δ8-desaturase or Δ5-, Δ6- or Δ9-elongase activity, a wide range of polyunsaturated fatty acids can be produced in the process according to the invention. Depending on the choice of the organisms, such as the advantageous plants, used for the process according to the invention, mixtures of the various polyunsaturated fatty acids or individual polyunsaturated fatty acids, such as EPA or ARA, can be produced in free or bound form. Depending on the prevailing fatty acid composition in the starting plant (C18:2- or C18:3-fatty acids), fatty acids which are derived from C18:2-fatty acids, such as GLA, DGLA or ARA, or fatty acids which are derived from C18:3-fatty acids, such as SDA, ETA or EPA, are thus obtained. If only linoleic acid (= LA, C18:$2^{\Delta 9,12}$) is present as unsaturated fatty acid in the plant used for the process, the process can only afford GLA, DGLA and ARA as products, all of which can be present as free fatty acids or in bound form. If only α-linolenic acid (= ALA, C18:$3^{\Delta 9,12,15}$) is present as unsaturated fatty acid in the plant used for the process, as is the case, for example, in linseed, the process can only afford SDA, ETA and EPA as products, all of which can be present as free fatty acids or in bound form, as described above. Owing to the modification of the activity of the enzymes involved in the synthesis, acyl-CoA:lysophospholipid acyltransferase, advantageously in combination with Δ5-, Δ6-desaturase and Δ6-elongase or with Δ5-Δ8-desaturase and Δ9-elongase or in combination with only the first two genes, Δ6-desaturase and Δ6-elongase or Δ8-desaturase and Δ9-elongase, of the synthesis cascade, it is possible to produce, in a targeted fashion, only individual products in the abovementioned organisms, advantageously in the abovementioned plants. Owing to the activity of Δ6-desaturase and Δ6-elongase, for example, GLA and

DGLA, or SDA and ETA, are formed, depending on the starting plant and unsaturated fatty acid. DGLA or ETA or mixtures of these are preferably formed. If Δ5-desaturase is additionally introduced into the organisms, advantageously into the plant, ARA or EPA is additionally formed. This also applies to organisms into which Δ8-desaturase and Δ9-elongase have been introduced previously. Advantageously, only ARA or EPA or mixtures of these are synthesized, depending on the fatty acid present in the organism, or in the plant, which acts as starting substance for the synthesis. Since biosynthetic cascades are involved, the end products in question are not present in pure form in the organisms. Small amounts of the precursor compounds are always additionally present in the end product. These small amounts amount to less than 20% by weight, advantageously less than 15% by weight, especially advantageously less than 10% by weight, most advantageously less than 5, 4, 3, 2 or 1% by weight, based on the end products DGLA, ETA or their mixtures, or ARA, EPA or their mixtures.

To increase the yield in the above-described process for the production of oils and/or triglycerides with an advantageously elevated content of polyunsaturated fatty acids, it is advantageous to increase the amount of starting product for the synthesis of fatty acids; this can be achieved for example by introducing, into the organism, a nucleic acid which encodes a polypeptide with Δ12-desaturase activity. This is particularly advantageous in oil-producing organisms such as oilseed rape which are high in oleic acid. Since these organisms are only low in linoleic acid (Mikoklajczak et al., Journal of the American Oil Chemical Society, 38, 1961, 678 - 681), the use of the abovementioned Δ12-desaturases for producing the starting material linoleic acid is advantageous.

Nucleic acids used in the process according to the invention are advantageously derived from plants such as algae such as Isochrysis or Crypthecodinium, algae/diatoms such as Phaeodactylum, mosses such as Physcomitrella or Ceratodon, or higher plants such as the Primulaceae such as Aleuritia, Calendula stellata, Osteospermum spinescens or Osteospermum hyoseroides, microorganisms such as fungi, such as Aspergillus, Thraustochytrium, Phytophthora, Entomophthora, Mucor or Mortierella, yeasts or animals such as nematodes such as Caenorhabditis, insects or humans. The nucleic acids are advantageously derived from fungi, animals, or from plants such as algae or mosses, preferably from nematodes such as Caenorhabditis.

The process according to the invention advantageously employs the abovementioned nucleic acid sequences or their derivatives or homologs which encode polypeptides which retain the enzymatic activity of the proteins encoded by nucleic acid sequences. These sequences, individually or in combination with the nucleic acid sequence which encode acyl-CoA:lysophospholipid acyltransferase, are cloned into expression constructs and used for the introduction into, and expression in, organisms. Owing to their construction, these expression constructs make possible an advantageous optimal synthesis of the polyunsaturated fatty acids produced in the process according to the invention.

In a preferred embodiment, the process furthermore comprises the step of obtaining a cell or an intact organism which comprises the nucleic acid sequences used in the process, where the cell and/or the organism is transformed with the nucleic acid sequence according to the invention which encodes the acyl-CoA:lysophospholipid acyltransferase, a gene construct or a vector as described above, alone or in combination with further nucleic acid sequences which encode proteins of the fatty acid or lipid metabolism. In a further preferred embodiment, this process furthermore comprises the step of obtaining the fine chemical from the culture. The culture can, for example, take the form of a fermentation culture, for example in the case of the cultivation of microorganisms, such as, for example, Mortierella, Saccharomyces or Thraustochytrium, or a greenhouse- or field-grown culture of a plant. The cell or the organism produced thus is advantageously a cell of an oil-producing organism, such as an oil crop, such as, for example, peanut, oilseed rape, canola, linseed, hemp, soybean, safflower, sunflowers or borage.

In the case of plant cells, plant tissue or plant organs, "growing" is understood as meaning, for example, the cultivation on or in a nutrient medium, or of the intact plant on or in a substrate, for example in a hydroponic culture, potting compost or on arable land.

For the purposes of the invention, "transgenic" or "recombinant" means with regard to, for example, a nucleic acid sequence, an expression cassette (= gene construct) or a vector comprising the nucleic acid sequence or an organism transformed with the nucleic acid sequences, expression cassette or vector according to the invention, all those constructions brought about by recombinant methods in which either

a) the nucleic acid sequence according to the invention, or

b) a genetic control sequence which is operably linked with the nucleic acid sequence according to the invention, for example a promoter, or

c) (a) and (b)

are not located in their natural genetic environment or have been modified by recombinant methods, it being possible for the modification to take the form of, for example, a substitution, addition, deletion, inversion or insertion of one or more nucleotide residues. The natural genetic environment is understood as meaning the natural genomic or chromosomal locus in the original organism or the presence in a genomic library. In the case of a genomic library, the natural genetic environment of the nucleic acid sequence is preferably retained, at least in part. The environment flanks the nucleic acid sequence at least on one side and has a sequence length of at least 50 bp, preferably at least 500 bp, especially preferably at least 1000 bp, most preferably at least 5000 bp. A naturally occurring expression cassette – for example the naturally occurring combination of the natural promoter of the inventive nucleic acid sequence with the corresponding acyl-CoA:lysophospholipid acyltransferase gene - becomes a transgenic expression cassette when this expression cassette is modified

by non-natural, synthetic ("artificial") methods such as, for example, mutagenic treatment. Suitable methods are described, for example, in US 5,565,350 or WO 00/15815.

A transgenic organism or transgenic plant for the purposes of the invention is understood as meaning, as above, that the nucleic acids used in the process are not at their natural locus in the genome of an organism, it being possible for the nucleic acids to be expressed homologously or heterologously. However, as mentioned, transgenic also means that, while the nucleic acids according to the invention are at their natural position in the genome of an organism, the sequence has been modified with regard to the natural sequence, and/or that the regulatory sequences of the natural sequences have been modified. Transgenic is preferably understood as meaning the expression of the nucleic acids according to the invention at an unnatural locus in the genome, i.e. homologous or, preferably, heterologous expression of the nucleic acids takes place. Preferred transgenic organisms are fungi such as Mortierella or plants such as the oil crops.

Organisms or host organisms for the nucleic acids, the expression cassette or the vector used in the process according to the invention are, in principle, advantageously all organisms which are capable of synthesizing fatty acids, specifically unsaturated fatty acids, and/or which are suitable for the expression of recombinant genes. Examples which may be mentioned are plants such as Arabidopsis, Asteraceae such as Calendula or crop plants such as soybean, peanut, castor-oil plant, sunflower, maize, cotton, flax, oilseed rape, coconut, oil palm, safflower (Carthamus tinctorius) or cacao bean, microorganisms, such as fungi, for example the genus Mortierella, Saprolegnia, or Pythium, bacteria, such as the genus Escherichia, yeasts, such as the genus Saccharomyces, cyanobacteria, ciliates, algae or protozoans such as dinoflagellates, such as Crypthecodinium. Preferred organisms are those which are naturally capable of synthesizing substantial amounts of oil, such as fungi, such as Mortierella alpina, Pythium insidiosum, or plants such as soybean, oilseed rape, coconut, oil palm, safflower, flax, hemp, castor-oil plant, Calendula, peanut, cacao bean or sunflower, or yeasts such as Saccharomyces cerevisiae with soybean, flax, oilseed rape, safflower, sunflower, Calendula, Mortierella or Saccharomyces cerevisiae being especially preferred. In principle, host organisms are, in addition to the abovementioned transgenic organisms, also transgenic animals, advantageously nonhuman animals, for example C. elegans.

Further utilizable host cells are detailed in: Goeddel, Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA (1990).

Expression strains which can be used, for example those with a lower protease activity, are described in: Gottesman, S., Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, California (1990) 119-128.

These include plant cells and certain tissues, organs and parts of plants in all their phenotypic forms such as anthers, fibers, root hairs, stalks, embryos, calli, cotyledons,

petioles, harvested material, plant tissue, reproductive tissue and cell cultures which are derived from the actual transgenic plant and/or can be used for bringing about the transgenic plant.

Transgenic plants which comprise the polyunsaturated fatty acids synthesized in the process according to the invention can advantageously be marketed directly without there being any need for the oils, lipids or fatty acids synthesized to be isolated. Plants for the process according to the invention are listed as meaning intact plants and all plant parts, plant organs or plant parts such as leaf, stem, seeds, root, tubers, anthers, fibers, root hairs, stalks, embryos, calli, cotyledons, petioles, harvested material, plant tissue, reproductive tissue and cell cultures which are derived from the actual transgenic plant and/or can be used for bringing about the transgenic plant. In this context, the seed comprises all parts of the seed such as the seed coats, epidermal cells, seed cells, endosperm or embryonic tissue. However, the compounds produced in the process according to the invention can also be isolated from the organisms, advantageously plants, in the form of their oils, fat, lipids and/or free fatty acids. Polyunsaturated fatty acids produced by this process can be obtained by harvesting the organisms, either from the crop in which they grow, or from the field. This can be done via pressing or extraction of the plant parts, preferably the plant seeds. In this context, the oils, fats, lipids and/or free fatty acids can be obtained by what is known as cold-beating or cold-pressing without applying heat by pressing. To allow for greater ease of disruption of the plant parts, specifically the seeds, they are previously comminuted, steamed or roasted. The seeds which have been pretreated in this manner can subsequently be pressed or extracted with solvents such as warm hexane. The solvent is subsequently removed. In the case of microorganisms, the latter are, after harvesting, for example extracted directly without further processing steps or else, after disruption, extracted via various methods with which the skilled worker is familiar. In this manner, more than 96% of the compounds produced in the process can be isolated. Thereafter, the resulting products are processed further, i.e. refined. In this process, substances such as the plant mucilages and suspended matter are first removed. What is known as desliming can be effected enzymatically or, for example, chemico-physically by addition of acid such as phosphoric acid. Thereafter, the free fatty acids are removed by treatment with a base, for example sodium hydroxide solution. The resulting product is washed thoroughly with water to remove the alkali remaining in the product and then dried. To remove the pigments remaining in the product, the products are subjected to bleaching, for example using filler's earth or active charcoal. At the end, the product is deodorized, for example using steam.

The PUFAs or LCPUFAs produced by this process are advantageously C_{18}-, C_{20}- or C_{22}-fatty acid molecules with at least two double bonds in the fatty acid molecule, preferably three, four, five or six double bonds. These C_{18}-, C_{20}- or C_{22}-fatty acid molecules can be isolated from the organism in the form of an oil, a lipid or a free fatty acid. Suitable organisms are, for example, those mentioned above. Preferred organisms are transgenic plants.

One embodiment of the invention is therefore oils, lipids or fatty acids or fractions thereof which have been produced by the above-described process, especially preferably oil, lipid or a fatty acid composition comprising PUFAs and being derived from transgenic plants.

A further embodiment according to the invention is the use of the oil, lipid, the fatty acids and/or the fatty acid composition in feedstuffs, foodstuffs, cosmetics or pharmaceuticals.

The term "oil", "lipid" or "fat" is understood as meaning a fatty acid mixture comprising unsaturated or saturated, preferably esterified, fatty acid(s). The oil, lipid or fat is preferably high in polyunsaturated free or, advantageously, esterified fatty acid(s), in particular linoleic acid, γ-linolenic acid, dihomo-γ-linolenic acid, arachidonic acid, α-linolenic acid, stearidonic acid, eicosatetraenoic acid, eicosapentaenoic acid, docosapentaenoic acid or docosahexaenoic acid. The content of unsaturated esterified fatty acids preferably amounts to approximately 30%, a content of 50% is more preferred, a content of 60%, 70%, 80% or more is even more preferred. For the analysis, the fatty acid content can, for example, be determined by gas chromatography after converting the fatty acids into the methyl esters by transesterification. The oil, lipid or fat can comprise various other saturated or unsaturated fatty acids, for example calendulic acid, palmitic acid, palmitoleic acid, stearic acid, oleic acid and the like. The content of the various fatty acids in the oil or fat can vary, in particular depending on the starting organism.

The polyunsaturated fatty acids with advantageously at least two double bonds which are produced in the process are, as described above, for example sphingolipids, phosphoglycerides, lipids, glycolipids, phospholipids, monoacylglycerol, diacylglycerol, triacylglycerol or other fatty acid esters.

Starting from the polyunsaturated fatty acids with advantageously at least two double bonds, which acids have been prepared in the process according to the invention, the polyunsaturated fatty acids which are present can be liberated for example via treatment with alkali, for example aqueous KOH or NaOH, or acid hydrolysis, advantageously in the presence of an alcohol such as methanol or ethanol, or via enzymatic cleavage, and isolated via, for example, phase separation and subsequent acidification via, for example, H_2SO_4. The fatty acids can also be liberated directly without the above-described processing step.

After their introduction into an organism, advantageously a plant cell or plant, the nucleic acids used in the process can either be present on a separate plasmid or integrated into the genome of the host cell. In the case of integration into the genome, integration can be random or else be effected by recombination such that the native gene is replaced by the copy introduced, whereby the production of the desired compound by the cell is modulated, or by the use of a gene in trans, so that the gene is linked operably with a functional expression unit which comprises at least one sequence which ensures the expression of a gene and at least one sequence which

ensures the polyadenylation of a functionally transcribed gene. The nucleic acids are advantageously introduced into the organisms via multiexpression cassettes or constructs for multiparallel expression, advantageously into the plants for the multiparallel seed-specific expression of genes.

Mosses and algae are the only known plant systems which produce substantial amounts of polyunsaturated fatty acids such as arachidonic acid (ARA) and/or eicosapentaenoic acid (EPA) and/or docosahexaenoic acid (DHA). Mosses comprise PUFAs in membrane lipids, while algae, organisms which are related to algae and a few fungi also accumulate substantial amounts of PUFAs in the triacylglycerol fraction. This is why nucleic acid molecules which are isolated from such strains which also accumulate PUFAs in the triacylglycerol fraction are particularly advantageous for the process according to the invention and thus for the modification of the lipid and PUFA production system in a host, in particular plants such as oil crops, for example oilseed rape, canola, linseed, hemp, soybeans, sunflowers and borage. They can therefore be used advantageously in the process according to the invention.

Substrates of the acyl-CoA:lysophospholipid acyltransferase(s) which are advantageously used are C_{16}-, C_{18}-, C_{20}- or C_{22}-fatty acids.

To produce the long-chain PUFAs according to the invention, the polyunsaturated C_{16}- or C_{18}-fatty acids must first be desaturated by the enzymatic activity of a desaturase and subsequently be elongated by at least two carbon atoms via an elongase. After one elongation cycle, this enzyme activity gives C_{18}- or C_{20}-fatty acids and after two or three elongation cycles C_{22}- or C_{24}-fatty acids. The activity of the desaturases and elongases used in the process according to the invention preferably leads to C_{18}-, C_{20}- and/or C_{22}-fatty acids, advantageously with at least two double bonds in the fatty acid molecule, preferably with three, four or five double bonds, especially preferably to give C_{20}- and/or C_{22}-fatty acids with at least two double bonds in the fatty acid molecule, preferably with three, four or five double bonds in the molecule. After a first desaturation and the elongation have taken place, further desaturation steps such as, for example, one in the $\Delta 5$ position may take place. Products of the process according to the invention which are especially preferred are dihomo-γ-linolenic acid, arachidonic acid, eicosapentaenoic acid, docosapentaenoic acid and/or docosahexaenoic acid. The C_{18}-fatty acids with at least two double bonds in the fatty acid can be elongated by the enzymatic activity according to the invention in the form of the free fatty acid or in the form of the esters, such as phospholipids, glycolipids, sphingolipids, phosphoglycerides, monoacylglycerol, diacylglycerol or triacylglycerol.

The preferred biosynthesis site of the fatty acids, oils, lipids or fats in the plants which are advantageously used is, for example, in general the seed or cell strata of the seed, so that seed-specific expression of the nucleic acids used in the process makes sense. However, it is obvious that the biosynthesis of fatty acids, oils or lipids need not be limited to the seed tissue, but can also take place in a tissue-specific manner in all the other parts of the plant, for example in epidermal cells or in the tubers.

If microorganisms such as yeasts, such as Saccharomyces or Schizosaccharomyces, fungi such as Mortierella, Aspergillus, Phytophtora, Entomophthora, Mucor or Thraustochytrium, algae such as Isochrysis, Phaeodactylum or Crypthecodinium are used as organisms in the process according to the invention, these organisms are advantageously grown in fermentation cultures.

Owing to the use of the nucleic acids according to the invention which encode acyl-CoA:lysophospholipid acyltransferase(s), the polyunsaturated fatty acids produced in the process can be increased by at least 10%, preferably by at least 15%, especially preferably by at least 20%, very especially preferably by at least 50% in comparison with the wild type of the organisms which do not comprise the nucleic acids recombinantly.

In principle, the polyunsaturated fatty acids produced by the process according to the invention in the organisms used in the process can be increased in two different ways. Advantageously, the pool of free polyunsaturated fatty acids and/or the content of the esterified polyunsaturated fatty acids produced via the process can be enlarged. Advantageously, the pool of esterified polyunsaturated fatty acids in the transgenic organisms is enlarged by the process according to the invention.

If microorganisms are used as organisms in the process according to the invention, they are grown or cultured in the manner with which the skilled worker is familiar, depending on the host organism. As a rule, microorganisms are grown in a liquid medium comprising a carbon source, usually in the form of sugars, a nitrogen source, usually in the form of organic nitrogen sources such as yeast extract or salts such as ammonium sulfate, trace elements such as salts of iron, manganese and magnesium and, if appropriate, vitamins, at temperatures of between 0°C and 100°C, preferably between 10°C and 60°C, while passing in oxygen. The pH of the liquid medium can either be kept constant, that is to say regulated during the culturing period, or not. The cultures can be grown batchwise, semi-batchwise or continuously. Nutrients can be provided at the beginning of the fermentation or fed in semicontinuously or continuously. The polyunsaturated fatty acids produced can be isolated from the organisms as described above by processes known to the skilled worker, for example by extraction, distillation, crystallization, if appropriate precipitation with salt, and/or chromatography. To this end, the organisms can advantageously be disrupted beforehand.

If the host organisms are microorganisms, the process according to the invention is advantageously carried out at a temperature of between 0°C and 95°C, preferably between 10°C and 85°C, especially preferably between 15°C and 75°C, very especially preferably between 15°C and 45°C.

In this process, the pH value is advantageously kept between pH 4 and 12, preferably between pH 6 and 9, especially preferably between pH 7 and 8.

The process according to the invention can be operated batchwise, semibatchwise or

continuously. An overview over known cultivation methods can be found in the textbook by Chmiel (Bioprozeßtechnik 1. Einführung in die Bioverfahrenstechnik [Bioprocess technology 1. Introduction to Bioprocess technology] (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook by Storhas (Bioreaktoren und periphere Einrichtungen [Bioreactors and peripheral equipment] (Vieweg Verlag, Braunschweig/Wiesbaden, 1994)).

The culture medium to be used must suitably meet the requirements of the strains in question. Descriptions of culture media for various microorganisms can be found in the textbook "Manual of Methods for General Bacteriology" of the American Society for Bacteriology (Washington D. C., USA, 1981).

As described above, these media which can be employed in accordance with the invention usually comprise one or more carbon sources, nitrogen sources, inorganic salts, vitamins and/or trace elements.

Preferred carbon sources are sugars, such as mono-, di- or polysaccharides. Examples of very good carbon sources are glucose, fructose, mannose, galactose, ribose, sorbose, ribulose, lactose, maltose, sucrose, raffinose, starch or cellulose. Sugars can also be added to the media via complex compounds such as molasses or other by-products from sugar raffination. The addition of mixtures of a variety of carbon sources may also be advantageous. Other possible carbon sources are oils and fats such as, for example, soya oil, sunflower oil, peanut oil and/or coconut fat, fatty acids such as, for example, palmitic acid, stearic acid and/or linoleic acid, alcohols and/or polyalcohols such as, for example, glycerol, methanol and/or ethanol, and/or organic acids such as, for example, acetic acid and/or lactic acid.

Nitrogen sources are usually organic or inorganic nitrogen compounds or materials comprising these compounds. Examples of nitrogen sources comprise ammonia in liquid or gaseous form or ammonium salts such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate or ammonium nitrate, nitrates, urea, amino acids or complex nitrogen sources such as cornsteep liquor, soya meal, soya protein, yeast extract, meat extract and others. The nitrogen sources can be used individually or as a mixture.

Inorganic salt compounds which may be present in the media comprise the chloride, phosphorus and sulfate salts of calcium, magnesium, sodium, cobalt, molybdenum, potassium, manganese, zinc, copper and iron.

Inorganic sulfur-containing compounds such as, for example, sulfates, sulfites, dithionites, tetrathionates, thiosulfates, sulfides, or else organic sulfur compounds such as mercaptans and thiols may be used as sources of sulfur for the production of sulfur-containing fine chemicals, in particular of methionine.

Phosphoric acid, potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding sodium-containing salts may be used as sources of phosphorus.

Chelating agents may be added to the medium in order to keep the metal ions in solution. Particularly suitable chelating agents include dihydroxyphenols such as catechol or protocatechuate and organic acids such as citric acid.

The fermentation media used according to the invention for culturing microorganisms usually also comprise other growth factors such as vitamins or growth promoters, which include, for example, biotin, riboflavin, thiamine, folic acid, nicotinic acid, panthothenate and pyridoxine. Growth factors and salts are frequently derived from complex media components such as yeast extract, molasses, cornsteep liquor and the like. It is moreover possible to add suitable precursors to the culture medium. The exact composition of the media compounds heavily depends on the particular experiment and is decided upon individually for each specific case. Information on the optimization of media can be found in the textbook "Applied Microbiol. Physiology, A Practical Approach" (Editors P.M. Rhodes, P.F. Stanbury, IRL Press (1997) pp. 53-73, ISBN 0 19 963577 3). Growth media can also be obtained from commercial suppliers, for example Standard 1 (Merck) or BHI (brain heart infusion, DIFCO) and the like.

All media components are sterilized, either by heat (20 min at 1.5 bar and 121°C) or by filter sterilization. The components may be sterilized either together or, if required, separately. All media components may be present at the start of the cultivation or added continuously or batchwise, as desired.

The culture temperature is normally between 15°C and 45°C, preferably at from 25°C to 40°C, and may be kept constant or may be altered during the experiment. The pH of the medium should be in the range from 5 to 8.5, preferably around 7.0. The pH for cultivation can be controlled during cultivation by adding basic compounds such as sodium hydroxide, potassium hydroxide, ammonia and aqueous ammonia or acidic compounds such as phosphoric acid or sulfuric acid. Foaming can be controlled by employing antifoams such as, for example, fatty acid polyglycol esters. To maintain the stability of plasmids it is possible to add to the medium suitable substances having a selective effect, for example antibiotics. Aerobic conditions are maintained by introducing oxygen or oxygen-containing gas mixtures such as, for example, ambient air into the culture. The temperature of the culture is normally 20° to 45°C and preferably 25°C to 40°C. The culture is continued until formation of the desired product is at a maximum. This aim is normally achieved within 10 to 160 hours.

The fermentation broths obtained in this way, in particular those containing polyunsaturated fatty acids, usually contain a dry mass of from 7.5 to 25% by weight.

The fermentation broth can then be processed further. The biomass may, according to requirement, be removed completely or partially from the fermentation broth by separation methods such as, for example, centrifugation, filtration, decanting or a combination of these methods or be left completely in said broth. It is advantageous to process the biomass after its separation.

However, the fermentation broth can also be thickened or concentrated without

separating the cells, using known methods such as, for example, with the aid of a rotary evaporator, thin-film evaporator, falling-film evaporator, by reverse osmosis or by nanofiltration. Finally, this concentrated fermentation broth can be processed to obtain the fatty acids present therein.

The fatty acids obtained in the process are also suitable as starting material for the chemical synthesis of further products of interest. For example, they can be used in combination with one another or alone for the preparation of pharmaceuticals, foodstuffs, animal feeds or cosmetics.

The invention furthermore relates to isolated nucleic acid sequences coding for polypeptides having acyl-CoA:lysophospholipid acyltransferase activity wherein the acyl-CoA:lysophospholipid acyltransferases encoded by said nucleic acid sequences specifically convert C_{16}-, C_{18}-, C_{20}- or C_{22}-fatty acids having at least one double bond in the fatty acid molecule.

Advantageous isolated nucleic acid sequences are sequences selected from the group consisting of:

a) a nucleic acid sequence having the sequence depicted in SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5 or SEQ ID NO: 7,

b) nucleic acid sequences which can be derived from the coding sequence comprised in SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5 or SEQ ID NO: 7 as a result of the degenerated genetic code

c) derivatives of the nucleic acid sequence depicted in SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5 or SEQ ID NO: 7 which code for polypeptides having the amino acid sequence depicted in SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6 or SEQ ID NO: 8 and are at least 40% homologous at the amino acid level to SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6 or SEQ ID NO: 8 and have an acyl-CoA:lysophospholipid-acyltransferase activity.

The abovementioned nucleic acid sequences are advantageously derived from a eukaryotic organism.

The nucleic acid sequences used in the process which code for proteins with acyl-CoA:lysophospholipid acyltransferase activity or for proteins of the fatty acid or lipid metabolism are advantageously introduced in an expression cassette (= nucleic acid construct) which makes possible the expression of the nucleic acids in an organism, advantageously a plant or a microorganism.

To introduce the nucleic acids used in the process, the latter are advantageously amplified and ligated in the known manner. Preferably, a procedure following the protocol for Pfu DNA polymerase or a Pfu/Taq DNA polymerase mixture is followed. The primers are selected taking into consideration the sequence to be amplified. The

primers should advantageously be chosen in such a way that the amplificate comprises the entire codogenic sequence from the start codon to the stop codon. After the amplification, the amplificate is expediently analyzed. For example, a gel-electrophoretic separation can be carried out, which is followed by a quantitative and a qualitative analysis. Thereafter, the amplificate can be purified following a standard protocol (for example Qiagen). An aliquot of the purified amplificate is then available for the subsequent cloning step. Suitable cloning vectors are generally known to the skilled worker. These include, in particular, vectors which are capable of replication in microbial systems, that is to say mainly vectors which ensure efficient cloning in yeasts or fungi and which make possible the stable transformation of plants. Those which must be mentioned in particular are various binary and cointegrated vector systems which are suitable for the T-DNA-mediated transformation. Such vector systems are, as a rule, characterized in that they comprise at least the vir genes required for the Agrobacterium-mediated transformation and the T-DNA-delimiting sequences (T-DNA border). These vector systems advantageously also comprise further cis-regulatory regions such as promoters and terminator sequences and/or selection markers, by means of which suitably transformed organisms can be identified. While in the case of cointegrated vector systems vir genes and T-DNA sequences are arranged on the same vector, binary systems are based on at least two vectors, one of which bears vir genes, but no T-DNA, while a second one bears T-DNA, but no vir gene. Owing to this fact, the last-mentioned vectors are relatively small, easy to manipulate and to replicate both in *E. coli* and in Agrobacterium. These binary vectors include vectors from the series pBIB-HYG, pPZP, pBecks, pGreen. In accordance with the invention, pBin19, pBI101, pBinAR, pGPTV and pCAMBIA are used by preference. An overview of the binary vectors and their use is found in Hellens et al, Trends in Plant Science (2000) 5, 446–451. In order to prepare the vectors, the vectors can first be linearized with restriction endonuclease(s) and then modified enzymatically in a suitable manner. Thereafter, the vector is purified, and an aliquot is employed for the cloning step. In the cloning step, the enzymatically cleaved and, if appropriate, purified amplificate is ligated with vector fragments which have been prepared in a similar manner, using ligase. In this context, a particular nucleic acid construct, or vector or plasmid construct, can have one or else more than one codogenic gene segment. The codogenic gene segments in these constructs are preferably linked operably with regulatory sequences. The regulatory sequences include, in particular, plant sequences such as the above-described promoters and terminator sequences. The constructs can advantageously be stably propagated in microorganisms, in particular in Escherichia coli and Agrobacterium tumefaciens, under selective conditions and make possible the transfer of heterologous DNA into plants or microorganisms.

The nucleic acids used in the process, the inventive nucleic acids and nucleic acid constructs, can be introduced into organisms such as microorganisms or advantageously plants, advantageously using cloning vectors, and thus be used in the transformation of plants such as those which are published and cited in: Plant Molecular Biology and Biotechnology (CRC Press, Boca Raton, Florida), Chapter 6/7, p. 71-119 (1993); F.F. White, Vectors for Gene Transfer in Higher Plants; in:

Transgenic Plants, Vol. 1, Engineering and Utilization, Ed.: Kung and R. Wu, Academic Press, 1993, 15-38; B. Jenes et al., Techniques for Gene Transfer, in: Transgenic Plants, Vol. 1, Engineering and Utilization, Ed.: Kung and R. Wu, Academic Press (1993), 128-143; Potrykus, Annu. Rev. Plant Physiol. Plant Molec. Biol. 42 (1991), 205-225. Thus, the nucleic acids, the inventive nucleic acids and nucleic acid constructs, and/or vectors used in the process can be used for the recombinant modification of a broad spectrum of organisms, advantageously plants, so that the latter become better and/or more efficient PUFA producers.

A series of mechanisms exists by which the modification of of an acyl-CoA:lyso-phospholipid acyltransferase protein can influence directly the yield, production and/or production efficiency of a fine chemical from an oil crop plant or a microorganism, owing to a modified protein. The number or activity of the acyl-CoA:lysophospholipid acyltransferase protein or gene and also of gene combinations of acyl-CoA:lyso-phospholipid acyltransferases, desaturases and/or elongases may have increased, so that greater amounts of the compounds produced are produced *de novo*, since the organisms lacked this activity and ability to biosynthesize prior to introduction of the corresponding gene(s). This applies analogously to the combination with further desaturases or elongases or further enzymes of the fatty acid and lipid metabolism. The use of various divergent sequences, i.e. sequences which differ at the DNA sequence level, may also be advantageous in this context, or else the use of promoters for gene expression which makes possible a different gene expression in the course of time, for example as a function of the degree of maturity of a seed or an oil-storing tissue.

Owing to the introduction of one or more acyl-CoA:lysophospholipid acyltransferase, desaturase and/or elongase genes into an organism, alone or in combination with other genes in a cell, it is not only possible to increase biosynthesis flux towards the end product, but also to increase, or to create *de novo* the corresponding triacylglycerol composition. Likewise, the number or activity of other genes which are involved in the import of nutrients which are required for the biosynthesis of one or more fine chemicals (e.g. fatty acids, polar and/or neutral lipids), can be increased, so that the concentration of these precursors, cofactors or intermediates within the cells or within the storage compartment is increased, whereby the ability of the cells to produce PUFAs as described below is enhanced further. Fatty acids and lipids are themselves desirable fine chemicals; by optimizing the activity or increasing the number of one or more acyl-CoA:lysophospholipid acyltransferases, desaturases and/or elongases which are involved in the biosynthesis of these compounds, or by destroying the activity of one or more desaturases which are involved in the degradation of these compounds, an enhanced yield, production and/or efficiency of production of fatty acid and lipid molecules in organisms, advantageously in plants, is made possible.

The isolated nucleic acid molecules used in the process according to the invention encode proteins or parts of these, where the proteins or the individual protein or parts thereof comprise(s) an amino acid sequence with sufficient homology to an amino acid

sequence which is shown in the sequence SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6 or SEQ ID NO: 8, so that the protein or part thereof retains an acyl-CoA:lyso-phospholipid acyltransferase activity. The protein or part thereof which is encoded by the nucleic acid molecule preferably retains its essential enzymatic activity and the ability to participate in the metabolism of compounds required for the synthesis of cell membranes or lipid bodies in organisms, advantageously in plants, or in the transport of molecules across these membranes. Advantageously, the protein encoded by the nucleic acid molecules is at least approximately 40%, preferably at least approximately 60% and more preferably at least approximately 70%, 80% or 90% and most preferably at least approximately 95%, 96%, 97%, 98%, 99% or more homologous to the amino acid sequences shown in SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6 or SEQ ID NO: 8. Advantageous embodiments of the inventive amino acid sequence of the sequence SEQ ID NO: 2 are amino acid sequences which have a valine residue instead of the methionine at position 30 of SEQ ID NO: 2 or have a glycine residue instead of the serine at position 100 or have a serine residue instead of the phenylalanine at position 170. These are indicated in SEQ ID NO: 4, SEQ ID NO: 6 and SEQ ID NO: 8, respectively.

Essential enzymatic activity of the acyl-CoA:lysophospholipid acyltransferases used is understood as meaning that they retain at least an enzymatic activity of at least 10%, preferably 20%, especially preferably 30% and very especially 40% in comparison with the proteins/enzymes encoded by the sequence with SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5 or SEQ ID NO: 7 and their derivatives and can thus participate in the metabolism of compounds required for the synthesis of fatty acids in an organism, advantageously a plant cell, or in the transport of molecules across membranes, meaning desaturated C_{16}-, C_{18}- or C_{20-24}-carbon chains with double bonds at at least two, advantageously three, four or five positions.

Nucleic acids which can advantageously be used in the process are derived from fungi or plants such as algae or mosses, such as the genera Physcomitrella, Thraustochytrium, Phytophthora, Ceratodon, Isochrysis, Aleurita, Muscarioides, Mortierella, Borago, Phaeodactylum, Crypthecodinium or from nematodes such as Caenorhabditis, specifically from the genera and species Physcomitrella patens, Phytophtora infestans, Ceratodon purpureus, Isochrysis galbana, Aleurita farinosa, Muscarioides viallii, Mortierella alpina, Borago officinalis, Phaeodactylum tricornutum, or especially advantageously from Caenorhabditis elegans.

Alternatively, the isolated nucleotide sequences used may encode acyl-CoA:lysophospholipid acyltransferases which hybridize with a nucleotide sequence of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5 or SEQ ID NO: 7, for example under stringent conditions.

The nucleic acid sequences used in the process are advantageously introduced into an expression cassette which makes possible the expression of the nucleic acids in organisms such as microorganisms or plants.

In doing so, the nucleic acid sequences which encode the acyl-CoA:lysophospholipid acyltransferases of the invention, the desaturases used and/or the elongases are linked operably with one or more regulatory signals, advantageously for enhancing gene expression. These regulatory sequences are intended to make possible the specific expression of the genes and proteins. Depending on the host organism, this may mean, for example, that the gene is expressed and/or overexpressed only after induction has taken place, or else that it expresses and/or overexpresses immediately. For example, these regulatory sequences take the form of sequences to which inductors or repressors bind, thus controlling the expression of the nucleic acid. In addition to these novel regulatory sequences, or instead of these sequences, the natural regulation of these sequences may still be present before the actual structural genes and, if appropriate, may have been genetically modified in such a way that natural regulation has been eliminated and expression of the genes has been enhanced. However, the expression cassette (= expression construct = gene construct) can also be simpler in construction, that is to say no additional regulatory signals have been inserted before the nucleic acid sequence or its derivatives, and the natural promoter together with its regulation was not removed. Instead, the natural regulatory sequence has been mutated in such a way that regulation no longer takes place and/or gene expression is enhanced. These modified promoters can also be positioned on their own before the natural gene in the form of part-sequences (= promoter with parts of the nucleic acid sequences used in accordance with the invention) in order to enhance the activity. Moreover, the gene construct may advantageously also comprise one or more what are known as enhancer sequences in operable linkage with the promoter, which make possible an enhanced expression of the nucleic acid sequence. Additional advantageous sequences, such as further regulatory elements or terminator sequences, may also be inserted at the 3' end of the DNA sequences. The acyl-CoA:lysophospholipid acyltransferase genes and the advantageously used Δ4-desaturase, Δ5-desaturase, Δ6-desaturase and/or Δ8-desaturase genes and/or Δ5-elongase, Δ6-elongase and/or Δ9-elongase genes may be present in one or more copies in the expression cassette (= gene construct). Preferably, only one copy of the genes is present in each expression cassette. This gene construct or the gene constructs can be expressed together in the host organism. In this context, the gene construct(s) can be inserted in one or more vectors and be present in the cell in free form, or else be inserted in the genome. It is advantageous for the insertion of further genes in the host genome when the genes to be expressed are present together in one gene construct.

In this context, the regulatory sequences or factors can, as described above, preferably have a positive effect on the gene expression of the genes introduced, thus enhancing it. Thus, an enhancement of the regulatory elements, advantageously at the transcriptional level, may take place by using strong transcription signals such as promoters and/or enhancers. In addition, however, enhanced translation is also possible, for example by improving the stability of the mRNA.

A further embodiment of the invention is one or more gene constructs which comprise

one or more sequences which are defined by SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5 or SEQ ID NO: 7 or its derivatives and which encode polypeptides as shown in SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6 or SEQ ID NO: 8. The abovementioned acyl-CoA:lysophospholipid acyltransferases lead advantageously to an exchange of fatty acids between the mono-, di- and/or triglyceride pool of the cell and the CoA-fatty acid ester pool, the substrate advantageously having one, two, three, four or five double bonds and advantageously 16, 18, 20, 22 or 24 carbon atoms in the fatty acid molecule. The same applies to their homologs, derivatives or analogs, which are linked operably with one or more regulatory signals, advantageously for enhancing gene expression.

Advantageous regulatory sequences for the novel process are present for example in promoters such as the cos, tac, trp, tet, trp-tet, lpp, lac, lpp-lac, laclq, T7, T5, T3, gal, trc, ara, SP6, λ-PR or λ-PL promoter and are advantageously employed in Gram-negative bacteria. Further advantageous regulatory sequences are, for example, present in the Gram-positive promoters amy and SPO2, in the yeast or fungal promoters ADC1, MFα, AC, P-60, CYC1, GAPDH, TEF, rp28, ADH or in the plant promoters CaMV/35S [Franck et al., Cell 21 (1980) 285–294], PRP1 [Ward et al., Plant. Mol. Biol. 22 (1993)], SSU, OCS, lib4, usp, STLS1, B33, nos or in the ubiquitin or phaseolin promoter. Advantageous in this context are also inducible promoters, such as the promoters described in EP-A-0 388 186 (benzenesulfonamide-inducible), Plant J. 2, 1992:397–404 (Gatz et al., tetracycline-inducible), EP–A–0 335 528 (abscissic acid-inducible) or WO 93/21334 (ethanol- or cyclohexenol-inducible) promoters. Further suitable plant promoters are the cytosolic FBPase promoter or the ST-LSI promoter of potato (Stockhaus et al., EMBO J. 8, 1989, 2445), the glycine max phosphoribosylpyrophosphate amidotransferase promoter (Genbank Accession No. U87999) or the node-specific promoter described in EP–A–0 249 676. Especially advantageous promoters are promoters which make possible the expression in tissues which are involved in the biosynthesis of fatty acids. Very especially advantageous are seed-specific promoters, such as the USP promoter as described, but also other promoters such as the LeB4, DC3, phaseolin or napin promoter. Further especially advantageous promoters are seed-specific promoters which can be used for monocotyledonous or dicotyledonous plants and which are described in US 5,608,152 (oilseed rape napin promoter), WO 98/45461 (Arabidopsis oleosin promoter), US 5,504,200 (Phaseolus vulgaris phaseolin promoter), WO 91/13980 (Brassica Bce4 promoter), by Baeumlein et al., Plant J., 2, 2, 1992:233–239 (LeB4 promoter from a legume), these promoters being suitable for dicots. Examples of promoters which are suitable for monocots are the barley lpt–2 or lpt–1 promoter (WO 95/15389 and WO 95/23230), the barley hordein promoter and other suitable promoters described in WO 99/16890.

In principle, it is possible to use all natural promoters together with their regulatory sequences, such as those mentioned above, for the novel process. It is also possible and advantageous to use synthetic promoters, either in addition or alone, in particular when they mediate seed-specific expression, such as those described in WO

99/16890.

In order to achieve a particularly high PUFA content, especially in transgenic plants, the PUFA biosynthesis genes should advantageously be expressed in oil crops in a seed-specific manner. To this end, seed-specific promoters can be used, or those promoters which are active in the embryo and/or in the endosperm. In principle, seed-specific promoters can be isolated both from dicotyledonous and from monocotyledonous plants. Preferred promoters are listed hereinbelow: USP (= unknown seed protein) and vicilin (Vicia faba) [Bäumlein et al., Mol. Gen Genet., 1991, 225(3)], napin (oilseed rape) [US 5,608,152], acyl carrier protein (oilseed rape) [US 5,315,001 and WO 92/18634], oleosin (Arabidopsis thaliana) [WO 98/45461 and WO 93/20216], phaseolin (Phaseolus vulgaris) [US 5,504,200], Bce4 [WO 91/13980], legumines B4 (LegB4 promoter) [Bäumlein et al., Plant J., 2,2, 1992], Lpt2 and lpt1 (barley) [WO 95/15389 and WO 95/23230], seed-specific promoters from rice, maize and wheat [WO 99/16890], Amy32b, Amy 6-6 and aleurain [US 5,677,474], Bce4 (oilseed rape) [US 5,530,149], glycinin (soybean) [EP 571 741], phosphoenol pyruvate carboxylase (soybean) [JP 06/62870], ADR12-2 (soybean) [WO 98/08962], isocitrate lyase (oilseed rape) [US 5,689,040] or α-amylase (barley) [EP 781 849].

Plant gene expression can also be facilitated via a chemically inducible promoter (see review in Gatz 1997, Annu. Rev. Plant Physiol. Plant Mol. Biol., 48:89-108). Chemically inducible promoters are particularly suitable when it is desired that gene expression should take place in a time-specific manner. Examples of such promoters are a salicylic-acid-inducible promoter (WO 95/19443), a tetracycline-inducible promoter (Gatz et al. (1992) Plant J. 2, 397-404) and an ethanol-inducible promoter.

To ensure the stable integration of the biosynthesis genes into the transgenic plant over a plurality of generations, each of the nucleic acids which encode acyl-CoA:lysophospholipid acyltransferase, the advantageous Δ4-desaturase, Δ5-desaturase, Δ6-desaturase, Δ8-desaturase and/or Δ5-elongase, Δ6-elongase and/or Δ9-elongase and which are used in the process should be expressed under the control of a separate promoter, preferably a promoter which differs from the other promoters, since repeating sequence motifs can lead to instability of the T-DNA, or to recombination events. In this context, the expression cassette is advantageously constructed in such a way that a promoter is followed by a suitable cleavage site, advantageously in a polylinker, for insertion of the nucleic acid to be expressed and, if appropriate, a terminator sequence is positioned behind the polylinker. This sequence is repeated several times, preferably three, four or five times, so that up to five genes can be combined in one construct and introduced into the transgenic plant in order to be expressed. Advantageously, the sequence is repeated up to three times. To express the nucleic acid sequences, the latter are inserted behind the promoter via the suitable cleavage site, for example in the polylinker. Advantageously, each nucleic acid sequence has its own promoter and, if appropriate, its own terminator sequence. However, it is also possible to insert a plurality of nucleic acid sequences behind a promoter and, if appropriate, before a terminator sequence. Here, the insertion site, or

the sequence, of the inserted nucleic acids in the expression cassette is not of critical importance, that is to say a nucleic acid sequence can be inserted at the first or last position in the cassette without its expression being substantially influenced thereby. Advantageously, different promoters such as, for example, the USP, LegB4 or DC3 promoter, and different terminator sequences can be used in the expression cassette. However, it is also possible to use only one type of promoter in the cassette. This, however, may lead to undesired recombination events.

As described above, the transcription of the genes which have been introduced should advantageously be terminated by suitable terminator sequences at the 3' end of the biosynthesis genes which have been introduced (behind the stop codon). An example of a sequence which can be used in this context is the OCS 1 terminator sequence. As is the case with the promoters, different terminator sequences should be used for each gene.

As described above, the gene construct can also comprise further genes to be introduced into the organisms. It is possible and advantageous to introduce into the host organisms, and to express therein, regulatory genes such as genes for inductors, repressors or enzymes which, owing to their enzyme activity, engage in the regulation of one or more genes of a biosynthesis pathway. These genes can be of heterologous or of homologous origin. Moreover, further biosynthesis genes of the fatty acid or lipid metabolism can advantageously be present in the nucleic acid construct, or gene construct; however, these genes can also be positioned on one or more further nucleic acid constructs. Biosynthesis genes of the fatty acid or lipid metabolism which are preferably used are a gene selected from the group consisting of acyl-CoA dehydrogenase(s), acyl-ACP [= acyl carrier protein] desaturase(s), acyl-ACP thioesterase(s), fatty acid acyltransferase(s), fatty acid synthase(s), fatty acid hydroxylase(s), acetyl-coenzyme A carboxylase(s), acyl-coenzyme A oxidase(s), fatty acid desaturase(s), fatty acid acetylenases, lipoxygenases, triacylglycerol lipases, allenoxide synthases, hydroperoxide lyases or fatty acid elongase(s) or combinations thereof. Especially advantageous nucleic acid sequences are biosynthesis genes of the fatty acid or lipid metabolism selected from the group of the Δ4-desaturase, Δ5-desaturase, Δ6-desaturase, Δ8-desaturase, Δ9-desaturase, Δ12-desaturase, Δ5-elongase, Δ6-elongase or Δ9-elongase.

In this context, the abovementioned desaturases can be cloned into expression cassettes of the invention in combination with other elongases and desaturases and used for transforming plants with the aid of Agrobacterium.

Here, the regulatory sequences or factors can, as described above, preferably have a positive effect on, and thus enhance, the expression of the genes which have been introduced. Thus, enhancement of the regulatory elements can advantageously take place at the transcriptional level by using strong transcription signals such as promoters and/or enhancers. However, an enhanced translation is also possible, for example by improving the stability of the mRNA. In principle, the expression cassettes

can be used directly for introduction into the plants or else be introduced into a vector.

These advantageous vectors, preferably expression vectors, comprise the nucleic acids which encode acyl-CoA:lysophospholipid acyltransferases and which are used in the process, or else a nucleic acid construct which comprises the nucleic acid used either alone or in combination with further biosynthesis genes of the fatty acid or lipid metabolism such as Δ4-desaturase, Δ5-desaturase, Δ6-desaturase, Δ8-desaturase, Δ9-desaturase, Δ12-desaturase, Δ5-elongase, Δ6-elongase and/or Δ9-elongase. As used in the present context, the term "vector" refers to a nucleic acid molecule which is capable of transporting another nucleic acid to which it is bound. One type of vector is a "plasmid", a circular double-stranded DNA loop into which additional DNA segments can be ligated. A further type of vector is a viral vector, it being possible for additional DNA segments to be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they have been introduced (for example bacterial vectors with bacterial replication origin). Other vectors are advantageously integrated into the genome of a host cell when they are introduced into the host cell, and thus replicate together with the host genome. Moreover, certain vectors can govern the expression of genes with which they are in operable linkage. These vectors are referred to in the present context as "expression vectors". Usually, expression vectors which are suitable for DNA recombination techniques take the form of plasmids. In the present description, "plasmid" and "vector" can be used exchangeably since the plasmid is the form of vector which is most frequently used. However, the invention is also intended to cover other forms of expression vectors, such as viral vectors, which exert similar functions. Furthermore, the term "vector" is also intended to encompass other vectors with which the skilled worker is familiar, such as phages, viruses such as SV40, CMV, TMV, transposons, IS elements, phasmids, phagemids, cosmids, linear or circular DNA.

The recombinant expression vectors advantageously used in the process comprise the nucleic acids described below or the above-described gene construct in a form which is suitable for expressing the nucleic acids used in a host cell, which means that the recombinant expression vectors comprise one or more regulatory sequences, selected on the basis of the host cells used for the expression, which regulatory sequence(s) is/are linked operably with the nucleic acid sequence to be expressed. In a recombinant expression vector, "linked operably" means that the nucleotide sequence of interest is bound to the regulatory sequence(s) in such a way that the expression of the nucleotide sequence is possible and they are bound to each other in such a way that both sequences carry out the predicted function which is ascribed to the sequence (for example in an in-vitro transcription/translation system, or in a host cell if the vector is introduced into the host cell). The term "regulatory sequence" is intended to comprise promoters, enhancers and other expression control elements (for example polyadenylation signals). These regulatory sequences are described, for example, in Goeddel: Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA (1990), or see: Gruber and Crosby, in: Methods in Plant Molecular Biology and Biotechnology, CRC Press, Boca Raton, Florida, Ed.: Glick and

Thompson, Chapter 7, 89-108, including the references cited therein. Regulatory sequences comprise those which govern the constitutive expression of a nucleotide sequence in many types of host cell and those which govern the direct expression of the nucleotide sequence only in specific host cells under specific conditions. The skilled worker knows that the design of the expression vector can depend on factors such as the choice of host cell to be transformed, the desired expression level of the protein and the like.

The recombinant expression vectors used can be designed for the expression of acyl-CoA:lysophospholipid acyltransferases, desaturases and elongases in prokaryotic or eukaryotic cells. This is advantageous since intermediate steps of the vector construction are frequently carried out in microorganisms for the sake of simplicity. For example, acyl-CoA:lysophospholipid acyltransferase, desaturase and elongase genes can be expressed in bacterial cells, insect cells (using Baculovirus expression vectors), yeast and other fungal cells (see Romanos, M.A., et al. (1992) "Foreign gene expression in yeast: a review", Yeast 8:423-488; van den Hondel, C.A.M.J.J., et al. (1991) "Heterologous gene expression in filamentous fungi", in: More Gene Manipulations in Fungi, J.W. Bennet & L.L. Lasure, Ed., pp. 396-428: Academic Press: San Diego; and van den Hondel, C.A.M.J.J., & Punt, P.J. (1991) "Gene transfer systems and vector development for filamentous fungi, in: Applied Molecular Genetics of Fungi, Peberdy, J.F., et al., Ed., pp. 1-28, Cambridge University Press: Cambridge), algae (Falciatore et al., 1999, Marine Biotechnology.1, 3:239-251), ciliates of the types: Holotrichia, Peritrichia, Spirotrichia, Suctoria, Tetrahymena, Paramecium, Colpidium, Glaucoma, Platyophrya, Potomacus, Desaturaseudocohnilembus, Euplotes, Engelmaniella and Stylonychia, in particular of the genus Stylonychia lemnae, using vectors in a transformation method as described in WO 98/01572 and, preferably, in cells of multi-celled plants (see Schmidt, R. and Willmitzer, L. (1988) "High efficiency Agrobacterium tumefaciens-mediated transformation of Arabidopsis thaliana leaf and cotyledon explants" Plant Cell Rep.: 583-586; Plant Molecular Biology and Biotechnology, C Press, Boca Raton, Florida, Chapter 6/7, pp. 71-119 (1993); F.F. White, B. Jenes et al., Techniques for Gene Transfer, in: Transgenic Plants, Vol. 1, Engineering and Utilization, Ed.: Kung and R. Wu, Academic Press (1993), 128-43; Potrykus, Annu. Rev. Plant Physiol. Plant Molec. Biol. 42 (1991), 205-225 (and references cited therein)). Suitable host cells are furthermore discussed in Goeddel, Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA (1990). As an alternative, the recombinant expression vector can be transcribed and translated in vitro, for example using T7-promoter regulatory sequences and T7-polymerase.

In most cases, the expression of proteins in prokaryotes involves the use of vectors comprising constitutive or inducible promoters which govern the expression of fusion or nonfusion proteins. Typical fusion expression vectors are, inter alia, pGEX (Pharmacia Biotech Inc; Smith, D.B., and Johnson, K.S. (1988) Gene 67:31-40), pMAL (New England Biolabs, Beverly, MA) and pRIT5 (Pharmacia, Piscataway, NJ), where glutathione S-transferase (GST), maltose-E binding protein and protein A, respectively,

is fused with the recombinant target protein.

Examples of suitable inducible nonfusion E. coli expression vectors are, inter alia, pTrc (Amann et al. (1988) Gene 69:301-315) and pET 11d (Studier et al., Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, California (1990) 60-89). The target gene expression from the pTrc vector is based on the transcription from a hybrid trp-lac fusion promoter by the host RNA polymerase. The target gene expression from the vector pET 11d is based on the transcription of a T7-gn10-lac fusion promoter, which is mediated by a viral RNA polymerase (T7 gn1), which is coexpressed. This viral polymerase is provided by the host strains BL21 (DE3) or HMS174 (DE3) from a resident λ-prophage which harbors a T7 gn1 gene under the transcriptional control of the lacUV 5 promoter.

Other vectors which are suitable for prokaryotic organisms are known to the skilled worker, these vectors are, for example in E. coli pLG338, pACYC184, the pBR series such as pBR322, the pUC series such as pUC18 or pUC19, the M113mp series, pKC30, pRep4, pHS1, pHS2, pPLc236, pMBL24, pLG200, pUR290, pIN-III113-B1, λgt11 or pBdCI, in Streptomyces pIJ101, pIJ364, pIJ702 or pIJ361, in Bacillus pUB110, pC194 or pBD214, in Corynebacterium pSA77 or pAJ667.

In a further embodiment, the expression vector is a yeast expression vector. Examples for vectors for expression in the yeast S. cerevisiae comprise pYeDesaturasec1 (Baldari et al. (1987) Embo J. 6:229-234), pMFa (Kurjan and Herskowitz (1982) Cell 30:933-943), pJRY88 (Schultz et al. (1987) Gene 54:113-123) and pYES2 (Invitrogen Corporation, San Diego, CA). Vectors and processes for the construction of vectors which are suitable for use in other fungi, such as the filamentous fungi, comprise those which are described in detail in: van den Hondel, C.A.M.J.J., & Punt, P.J. (1991) "Gene transfer systems and vector development for filamentous fungi, in: Applied Molecular Genetics of fungi, J.F. Peberdy et al., Ed., pp. 1-28, Cambridge University Press: Cambridge, or in: More Gene Manipulations in Fungi [J.W. Bennet & L.L. Lasure, Ed., pp. 396-428: Academic Press: San Diego]. Further suitable yeast vectors are, for example, pAG-1, YEp6, YEp13 or pEMBLYe23.

As an alternative, acyl-CoA:lysophospholipid acyltransferases, desaturases and/or elongases can be expressed in insect cells using Baculovirus expression vectors. Baculovirus vectors which are available for the expression of proteins in cultured insect cells (for example Sf9 cells) comprise the pAc series (Smith et al. (1983) Mol. Cell Biol. 3:2156-2165) and the pVL series (Lucklow and Summers (1989) Virology 170:31-39).

The abovementioned vectors offer only a small overview over suitable vectors which are possible. Further plasmids are known to the skilled worker and are described, for example, in: Cloning Vectors (Ed. Pouwels, P.H., et al., Elsevier, Amsterdam-New York-Oxford, 1985, ISBN 0 444 904018). For further suitable expression systems for prokaryotic and eukaryotic cells, see the Chapters 16 and 17 in Sambrook, J., Fritsch, E.F., and Maniatis, T., Molecular Cloning: A Laboratory Manual, 2nd edition, Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring

Harbor, NY, 1989.

In a further embodiment of the process, the acyl-CoA:lysophospholipid acyltransferases, desaturases and elongases can be expressed in single-celled plant cells (such as algae), see Falciatore et al., 1999, Marine Biotechnology 1 (3):239-251 and references cited therein, and in plant cells from higher plants (for example spermatophytes such as arable crops). Examples of plant expression vectors comprise those which are described in detail in: Becker, D., Kemper, E., Schell, J., and Masterson, R. (1992) "New plant binary vectors with selectable markers located proximal to the left border", Plant Mol. Biol. 20:1195-1197; and Bevan, M.W. (1984) "Binary Agrobacterium vectors for plant transformation", Nucl. Acids Res. 12:8711-8721; Vectors for Gene Transfer in Higher Plants; in: Transgenic Plants, Vol. 1, Engineering and Utilization, Ed.: Kung and R. Wu, Academic Press, 1993, p. 15-38.

A plant expression cassette preferably comprises regulatory sequences which are capable of governing the expression of genes in plant cells and which are linked operably so that each sequence can fulfill its function, such as transcriptional termination, for example polyadenylation signals. Preferred polyadenylation signals are those which are derived from Agrobacterium tumefaciens T-DNA, such as gene 3 of the Ti plasmid pTiACH5 (Gielen et al., EMBO J. 3 (1984) 835 et seq.), which is known as octopine synthase, or functional equivalents thereof, but all other terminator sequences which are functionally active in plants are also suitable.

Since plant gene expression is very often not limited to the transcriptional level, a plant expression cassette preferably comprises other sequences which are linked operably, such as translation enhancers, for example the overdrive sequence, which enhances the tobacco mosaic virus 5' – untranslated leader sequence, which increases the protein/RNA ratio (Gallie et al., 1987, Nucl. Acids Research 15:8693-8711).

As described above, plant gene expression must be linked operably with a suitable promoter which triggers gene expression with the correct timing or in a cell- or tissue-specific manner. Utilizable promoters are constitutive promoters (Benfey et al., EMBO J. 8 (1989) 2195-2202), such as those which are derived from plant viruses, such as 35S CaMV (Franck et al., Cell 21 (1980) 285-294), 19S CaMV (see also US 5352605 and WO 84/02913), or plant promoters, such as the promoter of the small rubisco subunit, which is described in US 4,962,028.

Other preferred sequences for use in operable linkage in plant gene expression cassettes are targeting sequences, which are required for steering the gene product into its corresponding cell compartment (see a review in Kermode, Crit. Rev. Plant Sci. 15, 4 (1996) 285-423 and references cited therein), for example into the vacuole, into the nucleus, all types of plastids, such as amyloplasts, chloroplasts, chromoplasts, the extracellular space, the mitochondria, the endoplasmid reticulum, elaioplasts, peroxisomes and other compartments of plant cells.

As described above, plant gene expression can also be achieved via a chemically

inducible promoter (see review in Gatz 1997, Annu. Rev. Plant Physiol. Plant Mol. Biol., 48:89-108). Chemically inducible promoters are particularly suitable when it is desired that the gene expression takes place in a time-specific manner. Examples of such promoters are a salicylic-acid-inducible promoter (WO 95/19443), a tetracyclin-inducible promoter (Gatz et al. (1992) Plant J. 2, 397-404) and an ethanol-inducible promoter.

Promoters which respond to biotic or abiotic stress conditions are also suitable, for example the pathogen-induced PRP1 gene promoter (Ward et al., Plant. Mol. Biol. 22 (1993) 361-366), the heat-inducible tomato hsp80 promoter (US 5,187,267), the chill-inducible potato alpha-amylase promoter (WO 96/12814) or the wound-inducible pinII promoter (EP-A-0 375 091).

Especially preferred are those promoters which bring about the gene expression in tissues and organs in which the biosynthesis of fatty acids, lipids and oils takes place, in seed cells, such as cells of the endosperm and of the developing embryo. Suitable promoters are the oilseed rape napin gene promoter (US 5,608,152), the Vicia faba USP promoter (Baeumlein et al., Mol Gen Genet, 1991, 225 (3):459-67), the Arabidopsis oleosin promoter (WO 98/45461), the Phaseolus vulgaris phaseolin promoter (US 5,504,200), the Brassica Bce4 promoter (WO 91/13980) or the legumine B4 promoter (LeB4; Baeumlein et al., 1992, Plant Journal, 2 (2):233-9), and promoters which bring about the seed-specific expression in monocotyledonous plants such as maize, barley, wheat, rye, rice and the like. Suitable noteworthy promoters are the barley lpt2 or lpt1 gene promoter (WO 95/15389 and WO 95/23230) or the promoters from the barley hordein gene, the rice glutelin gene, the rice oryzin gene, the rice prolamine gene, the wheat gliadine gene, the wheat glutelin gene, the maize zeine gene, the oat glutelin gene, the sorghum kasirin gene or the rye secalin gene, which are described in WO 99/16890.

In particular, it may be desired to bring about the multiparallel expression of the acyl-CoA:lysophospholipid acyltransferases used in the process alone or in combination with desaturases and/or elongases. Such expression cassettes can be introduced via the simultaneous transformation of a plurality of individual expression constructs or, preferably, by combining a plurality of expression cassettes on one construct. Also, a plurality of vectors can be transformed with in each case a plurality of expression cassettes and then transferred into the host cell.

Other promoters which are likewise especially suitable are those which bring about plastid-specific expression, since plastids constitute the compartment in which the precursors and some end products of lipid biosynthesis are synthesized. Suitable promoters, such as the viral RNA polymerase promoter, are described in WO 95/16783 and WO 97/06250, and the clpP promoter from Arabidopsis, described in WO 99/46394.

Vector DNA can be introduced into prokaryotic or eukaryotic cells via conventional transformation or transfection techniques. The terms "transformation" and "transfection", conjugation and transduction, as used in the present context, are

intended to comprise a multiplicity of methods known in the prior art for the introduction of foreign nucleic acid (for example DNA) into a host cell, including calcium phosphate or calcium chloride coprecipitation, DEAE-dextran-mediated transfection, lipofection, natural competence, chemically mediated transfer, electroporation or particle bombardment. Suitable methods for the transformation or transfection of host cells, including plant cells, can be found in Sambrook et al. (Molecular Cloning: A Laboratory Manual., 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989) and other laboratory textbooks such as Methods in Molecular Biology, 1995, Vol. 44, Agrobacterium protocols, Ed.: Gartland and Davey, Humana Press, Totowa, New Jersey.

Host cells which are suitable in principle for taking up the nucleic acid according to the invention, the gene product according to the invention or the vector according to the invention are all prokaryotic or eukaryotic organisms. The host organisms which are advantageously used are microorganisms such as fungi or yeasts, or plant cells, preferably plants or parts thereof. Fungi, yeasts or plants are preferably used, especially preferably plants, very especially preferably plants such as oil crops, which are high in lipid compounds, such as oilseed rape, evening primrose, hemp, thistle, peanut, canola, linseed, soybean, safflower, sunflower, borage, or plants such as maize, wheat, rye, oats, triticale, rice, barley, cotton, cassava, pepper, Tagetes, Solanacea plants such as potato, tobacco, eggplant and tomato, Vicia species, pea, alfalfa, bushy plants (coffee, cacao, tea), Salix species, trees (oil palm, coconut), and perennial grasses and fodder crops. Especially preferred plants according to the invention are oil crops such as soybean, peanut, oilseed rape, canola, linseed, hemp, evening primrose, sunflower, safflower, trees (oil palm, coconut).

The invention furthermore relates to isolated nucleic acid sequences as described above coding for polypeptides having acyl-CoA:lysophospholipid-acyltransferase activity, wherein the acyl-CoA:lysophospholipid acyltransferases encoded by said nucleic acid sequences specifically convert C_{16}-, C_{18}-, C_{20}- or C_{22}-fatty acids having at least one double bond in the fatty acid molecule.

Advantageous isolated nucleic acid sequences are sequences selected from the group consisting of:

d) a nucleic acid sequence having the sequence depicted in SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5 or SEQ ID NO: 7,

e) nucleic acid sequences which can be derived from the coding sequence comprised in SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5 or SEQ ID NO: 7 as a result of the degenerated genetic code

f) derivatives of the nucleic acid sequence depicted in SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5 or SEQ ID NO: 7 which code for polypeptides having the amino acid sequence depicted in SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6 or SEQ ID NO: 8 and are at least 40% homologous at the amino acid level to

SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6 or SEQ ID NO: 8 and have an acyl-CoA:lysophospholipid-acyltransferase activity.

The abovementioned nucleic acids according to the invention are derived from organisms such as animals, ciliates, fungi, plants such as algae or dinoflagellates which are capable of synthesizing PUFAs.

In an advantageous embodiment, the term "nucleic acid (molecule)" as used in the present context additionally comprises the untranslated sequence at the 3' and at the 5' end of the coding gene region: at least 500, preferably 200, especially preferably 100 nucleotides of the sequence upstream of the 5' end of the coding region and at least 100, preferably 50, especially preferably 20 nucleotides of the sequence downstream of the 3' end of the coding gene region. An "isolated" nucleic acid molecule is separate from other nucleic acid molecules which are present in the natural source of the nucleic acid. An "isolated" nucleic acid preferably has no sequences which naturally flank the nucleic acid in the genomic DNA of the organism from which the nucleic acid is derived (for example sequences which are located at the 5' and 3' ends of the nucleic acid). In various embodiments, the isolated acyl-CoA:lysophospholipid acyltransferase molecule can comprise for example fewer than approximately 5 kb, 4 kb, 3 kb, 2 kb, 1 kb, 0.5 kb or 0.1 kb of nucleotide sequences which naturally flank the nucleic acid molecule in the genomic DNA of the cell from which the nucleic acid is derived.

The nucleic acid molecules used in the process, for example a nucleic acid molecule with a nucleotide sequence of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5 or SEQ ID NO: 7 or of a part thereof can be isolated using molecular-biological standard techniques and the sequence information provided herein. Also, for example a homologous sequence or homologous, conserved sequence regions can be identified at the DNA or amino acid level with the aid of comparative algorithms. They can be used as hybridization probe together with standard hybridization techniques (such as, for example, those described in Sambrook et al., Molecular Cloning: A Laboratory Manual. 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989) for isolating further nucleic acid sequences which can be used in the process. Moreover, a nucleic acid molecule comprising a complete sequence of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5 or SEQ ID NO: 7 or a part thereof can be isolated by polymerase chain reaction, where oligonucleotide primers which are based on this sequence or on parts thereof are used (for example a nucleic acid molecule comprising the complete sequence or part thereof can be isolated by polymerase chain reaction using oligonucleotide primers which have been generated based on this same sequence). For example, mRNA can be isolated from cells (for example by means of the guanidinium thiocyanate extraction method of Chirgwin et al. (1979) Biochemistry 18:5294-5299) and cDNA by means of reverse transcriptase (for example Moloney MLV reverse transcriptase, available from Gibco/BRL, Bethesda, MD, or AMV reverse transcriptase, available from Seikagaku America, Inc., St. Petersburg, FL). Synthetic oligonucleotide primers for the amplification by means of polymerase chain reaction can be generated based on one of the sequences shown in

SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5 or SEQ ID NO: 7 or with the aid of the amino acid sequences detailed in SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6 or SEQ ID NO: 8. A nucleic acid according to the invention can be amplified by standard PCR amplification techniques using cDNA or, alternatively, genomic DNA as template and suitable oligonucleotide primers. The nucleic acid amplified thus can be cloned into a suitable vector and characterized by means of DNA sequence analysis. Oligonucleotides which correspond to a desaturase nucleotide sequence can be generated by standard synthetic methods, for example using an automatic DNA synthesizer.

Homologs of the acyl-CoA:lysophospholipid acyltransferase nucleic acid sequences with the sequence SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5 or SEQ ID NO: 7 means, for example, allelic variants with at least approximately 40 to 60%, preferably at least approximately from 60 to 70%, more preferably at least approximately from 70 to 80%, 80% to 90% or 90 to 95% and even more preferably at least approximately 95%, 96%, 97%, 98%, 99% or more homology with a nucleotide sequence shown in SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5 or SEQ ID NO: 7 or its homologs, derivatives or analogs or parts thereof. Furthermore, isolated nucleic acid molecules of a nucleotide sequence which hybridize with one of the nucleotide sequences shown in SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5 or SEQ ID NO: 7 or with a part thereof, for example hybridized under stringent conditions. Allelic variants comprise in particular functional variants which can be obtained by deletion, insertion or substitution of nucleotides from/into the sequence detailed in SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5 or SEQ ID NO: 7, it being intended, however, that the enzyme activity of the resulting proteins which are synthesized is advantageously retained for the insertion of one or more genes. Proteins which retain the enzymatic activity of acyl-CoA:lysophospholipid acyltransferase, i.e. whose activity is essentially not reduced, means proteins with at least 10%, preferably 20%, especially preferably 30%, very especially preferably 40% of the original enzyme activity in comparison with the protein encoded by SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5 or SEQ ID NO: 7.

Homologs of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5 or SEQ ID NO: 7 means for example also bacterial, fungal and plant homologs, truncated sequences, single-stranded DNA or RNA of the coding and noncoding DNA sequence.

Homologs of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5 or SEQ ID NO: 7 also mean derivatives such as, for example, promoter variants. The promoters upstream of the nucleotide sequences detailed can be modified by one or more nucleotide exchanges, by insertion(s) and/or deletion(s) without the functionality or activity of the promoters being adversely affected, however. It is furthermore possible that the modification of the promoter sequence enhances their activity or that they are replaced entirely by more active promoters, including those from heterologous organisms.

The abovementioned nucleic acids and protein molecules with acyl-CoA:lysophospholipid acyltransferase activity which are involved in the metabolism of lipids and fatty

acids, PUFA cofactors and enzymes or in the transport of lipophilic compounds across membranes are used in the process according to the invention for the modulation of the production of PUFAs in transgenic organisms, advantageously in plants, such as maize, wheat, rye, oats, triticale, rice, barley, soybean, peanut, cotton, Linum species such as linseed or flax, Brassica species such as oilseed rape, canola and turnip rape, pepper, sunflower, borage, evening primrose and Tagetes, Solanaceae plants such as potato, tobacco, eggplant and tomato, Vicia species, pea, cassava, alfalfa, bushy plants (coffee, cacao, tea), Salix species, trees (oil palm, coconut) and perennial grasses and fodder crops, either directly (for example when the overexpression or optimization of a fatty acid biosynthesis protein has a direct effect on the yield, production and/or production efficiency of the fatty acid from modified organisms) and/or can have an indirect effect which nevertheless leads to an enhanced yield, production and/or production efficiency of the PUFAs or a reduction of undesired compounds (for example when the modulation of the metabolism of lipids and fatty acids, cofactors and enzymes leads to modifications of the yield, production and/or production efficiency or the composition of the desired compounds within the cells, which, in turn, can affect the production of one or more fatty acids).

The combination of various precursor molecules and biosynthesis enzymes leads to the production of various fatty acid molecules, which has a decisive effect on lipid composition, since polyunsaturated fatty acids (= PUFAs) are not only incorporated into triacylglycerol but also into membrane lipids.

Lipid synthesis can be divided into two sections: the synthesis of fatty acids and their binding to sn-glycerol-3-phosphate, and the addition or modification of a polar head group. Usual lipids which are used in membranes comprise phospholipids, glycolipids, sphingolipids and phosphoglycerides. Fatty acid synthesis starts with the conversion of acetyl-CoA into malonyl-CoA by acetyl-CoA carboxylase or into acetyl-ACP by acetyl transacylase. After a condensation reaction, these two product molecules together form acetoacetyl-ACP, which is converted via a series of condensation, reduction and dehydratization reactions so that a saturated fatty acid molecule with the desired chain length is obtained. The production of the unsaturated fatty acids from these molecules is catalyzed by specific desaturases, either aerobically by means of molecular oxygen or anaerobically (regarding the fatty acid synthesis in microorganisms, see F.C. Neidhardt et al. (1996) E. coli and Salmonella. ASM Press: Washington, D.C., pp. 612-636 and references cited therein; Lengeler et al. (Ed.) (1999) Biology of Procaryotes. Thieme: Stuttgart, New York, and the references therein, and Magnuson, K., et al. (1993) Microbiological Reviews 57:522-542 and the references therein). To undergo the further elongation steps, the resulting phospholipid-bound fatty acids must be returned to the fatty acid CoA ester pool. This is made possible by acyl-CoA:lysophospholipid acyltransferases. Moreover, these enzymes are capable of transferring the elongated fatty acids from the CoA esters back to the phospholipids. If appropriate, this reaction sequence can be followed repeatedly (see figure 10).

Examples of precursors for the biosynthesis of PUFAs are oleic acid, linoleic acid and

linolenic acid. These C_{18}-carbon fatty acids must be elongated to C_{20} and C_{22} in order to obtain fatty acids of the eicosa and docosa chain type. With the aid of the acyl-CoA:lysophospholipid acyltransferases used in the process, advantageous in combination with desaturases such as Δ4-, Δ5-, Δ6- and Δ8-desaturases and/or Δ5-, Δ6-, Δ9-elongases, arachidonic acid, eicosapentaenoic acid, docosapentaenoic acid or docosahexaenoic acid and various other long-chain PUFAs can be obtained, extracted and employed in various applications regarding foodstuffs, feedstuffs, cosmetics or pharmaceuticals. Preferably C_{18}-, C_{20}- and/or C_{22}-fatty acids with at least two, advantageously at least three, four, five or six, double bonds in the fatty acid molecule can be prepared using the abovementioned enzymes, to give preferably C_{20}- or C_{22}-fatty acids with advantageously three, four or five double bonds in the fatty acid molecule. Desaturation may take place before or after elongation of the fatty acid in question. This is why the products of the desaturase activities and the further desaturation and elongation steps which are possible result in preferred PUFAs with a higher degree of desaturation, including a further elongation from C_{20}- to C_{22}-fatty acids, to fatty acids such as γ-linolenic acid, dihomo-γ-linolenic acid, arachidonic acid, stearidonic acid, eicosatetraenoic acid or eicosapentaenoic acid. Substrates of the acyl-CoA: lysophospholipid acyltransferases used in the process according to the invention are C_{16}-, C_{18}-, C_{20}- or C_{22}-fatty acids such as, for example, palmitic acid, palmitoleic acid, linoleic acid, γ-linolenic acid, α-linolenic acid, dihomo-γ-linolenic acid, eicosatetraenoic acid or stearidonic acid. Preferred substrates are linoleic acid, γ-linolenic acid and/or α-linolenic acid, dihomo-γ-linolenic acid, arachidonic acid, eicosatetraenoic acid or eicosapentaenoic acid. The C_{18}-, C_{20}- or C_{22}-fatty acids with at least two double bonds in the fatty acid are obtained in the process according to the invention in the form of the free fatty acid or in the form of their esters, for example in the form of their glycerides.

The term "glyceride" is understood as meaning glycerol esterified with one, two or three carboxyl radicals (mono-, di- or triglyceride). "Glyceride" is also understood as meaning a mixture of various glycerides. The glyceride or glyceride mixture may comprise further additions, for example free fatty acids, antioxidants, proteins, carbohydrates, vitamins and/or other substances.

For the purposes of the invention, a "glyceride" is furthermore understood as meaning glycerol derivatives. In addition to the above-described fatty acid glycerides, these also include glycerophospholipids and glyceroglycolipids. Preferred examples which may be mentioned in this context are the glycerophospholipids such as lecithin (phosphatidylcholine), cardiolipin, phosphatidylglycerol, phosphatidylserine and alkylacylglycerophospholipids.

Furthermore, fatty acids must subsequently be translocated to various modification sites and incorporated into the triacylglycerol storage lipid. A further important step in lipid synthesis is the transfer of fatty acids to the polar head groups, for example by glycerol fatty acid acyltransferase (see Frentzen, 1998, Lipid, 100(4-5):161-166).

Publications on plant fatty acid biosynthesis and on the desaturation, the lipid metabolism and the membrane transport of lipidic compounds, on beta-oxidation, fatty acid modification and cofactors, triacylglycerol storage and triacylglycerol assembly, including the references therein, see the following papers: Kinney, 1997, Genetic Engineering, Ed.: JK Setlow, 19:149-166; Ohlrogge and Browse, 1995, Plant Cell 7:957-970; Shanklin and Cahoon, 1998, Annu. Rev. Plant Physiol. Plant Mol. Biol. 49:611-641; Voelker, 1996, Genetic Engineering, Ed.: JK Setlow, 18:111-13; Gerhardt, 1992, Prog. Lipid R. 31:397-417; Gühnemann-Schäfer & Kindl, 1995, Biochim. Biophys Acta 1256:181-186; Kunau et al., 1995, Prog. Lipid Res. 34:267-342; Stymne et al., 1993, in: Biochemistry and Molecular Biology of Membrane and Storage Lipids of Plants, Ed.: Murata and Somerville, Rockville, American Society of Plant Physiologists, 150-158, Murphy & Ross 1998, Plant Journal. 13(1):1-16.

The PUFAs produced in the process comprise a group of molecules which higher animals are no longer capable of synthesizing and must therefore take up, or which higher animals are no longer capable of synthesizing themselves in sufficient quantity and must therefore take up additional quantities, although they can be synthesized readily by other organisms such as bacteria; for example, cats are no longer capable of synthesizing arachidonic acid.

The term "acyl-CoA:lysophospholipid acyltransferases" comprises for the purposes of the invention proteins which participate in the transfer of the fatty acids bound to phospholipids to the CoA-ester pool and vice versa and their homologs, derivatives and analogs. Phospholipids for the purposes of the invention are understood as meaning phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylglycerol and/or phosphatidylinositol, advantageously phosphatidylcholine. The terms acyl-CoA:lysophospholipid acyltransferase(s) comprise nucleic acid sequences which encode an acyl-CoA:lysophospholipid acyltransferase and part of which may be a coding region and likewise corresponding 5' and 3' untranslated sequence regions. The terms production or productivity are known in the art and encompass the concentration of the fermentation product (compounds of the formula I) which is formed within a specific period of time and in a specific fermentation volume (for example kg of product per hour per liter). The term production efficiency comprises the time required for obtaining a specific production quantity (for example the time required by the cell to establish a certain throughput rate of a fine chemical). The term yield or product/carbon yield is known in the art and comprises the efficiency of the conversion of the carbon source into the product (i.e. the fine chemical). This is usually expressed for example as kg of product per kg of carbon source. By increasing the yield or production of the compound, the amount of the molecules obtained of this compound, or of the suitable molecules of this compound obtained in a specific culture quantity over a specified period of time is increased. The terms biosynthesis or biosynthetic pathway are known in the art and comprise the synthesis of a compound, preferably an organic compound, by a cell from intermediates, for example in a multi-step and strongly regulated process. The terms catabolism or catabolic pathway are known in the art and comprise the cleavage of a compound, preferably of an organic

compound, by a cell to give catabolites (in more general terms, smaller or less complex molecules), for example in a multi-step and strongly regulated process. The term metabolism is known in the art and comprises the totality of the biochemical reactions which take place in an organism. The metabolism of a certain compound (for example the metabolism of a fatty acid) thus comprises the totality of the biosynthetic pathways, modification pathways and catabolic pathways of this compound in the cell which relate to this compound.

In a further embodiment, derivatives of the nucleic acid molecule according to the invention represented in SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5 or SEQ ID NO: 7 encode proteins with at least 40%, advantageously from approximately 50 to 60%, advantageously at least from approximately 60 to 70% and more preferably at least from approximately 70 to 80%, 80 to 90%, 90 to 95% and most preferably at least approximately 96%, 97%, 98%, 99% or more homology (= identity) with a complete amino acid sequence of SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6 or SEQ ID NO: 8. The homology was calculated over the entire amino acid or nucleic acid sequence region. The program PileUp (J. Mol. Evolution., 25, 351-360, 1987, Higgins et al., CABIOS, 5 1989: 151–153) or the programs Gap and BestFit [Needleman and Wunsch (J. Mol. Biol. 48; 443-453 (1970) and Smith and Waterman (Adv. Appl. Math. 2; 482-489 (1981)], which are part of the GCG software packet [Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711 (1991)], were used for the sequence alignment. The sequence homology values which are indicated above as a percentage were determined over the entire sequence region using the program BestFit and the following settings: Gap Weight: 8, Length Weight: 2.

Moreover, the invention comprises nucleic acid molecules which differ from one of the nucleotide sequences shown in SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5 or SEQ ID NO: 7 (and parts thereof) owing to the degeneracy of the genetic code and which thus encode the same acyl-CoA:lysophospholipid acyltransferase as those encoded by the nucleotide sequences shown in SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5 or SEQ ID NO: 7.

In addition to the acyl-CoA:lysophospholipid acyltransferase(s) shown in SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5 or SEQ ID NO: 7, the skilled worker will recognize that DNA sequence polymorphisms which lead to changes in the amino acid sequences of the acyl-CoA:lysophospholipid acyltransferase(s) may exist within a population. These genetic polymorphisms in the acyl-CoA:lysophospholipid acyltransferase gene may exist between individuals within a population owing to natural variation. These natural variants usually bring about a variance of 1 to 5% in the nucleotide sequence of the acyl-CoA:lysophospholipid acyltransferase gene. Each and every one of these nucleotide variations and resulting amino acid polymorphisms in the acyl-CoA:lysophospholipid acyltransferase which are the result of natural variation and do not modify the functional activity of acyl-CoA:lysophospholipid acyltransferases are to be encompassed by the invention.

Owing to their homology to the acyl-CoA:lysophospholipid acyltransferase nucleic acids disclosed here, nucleic acid molecules which are advantageous for the process according to the invention can be isolated following standard hybridization techniques under stringent hybridization conditions, using the sequences or part thereof as hybridization probe. In this context it is possible, for example, to use isolated nucleic acid molecules which are least 15 nucleotides in length and which hybridize under stringent conditions with the nucleic acid molecules which comprise a nucleotide sequence of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5 or SEQ ID NO: 7. Nucleic acids with at least 25, 50, 100, 250 or more nucleotides can also be used. The term "hybridizes under stringent conditions" as used in the present context is intended to describe hybridization and washing conditions under which nucleotide sequences with at least 60% homology to one another usually remain hybridized with one another. Conditions are preferably such that sequences with at least approximately 65%, preferably at least approximately 70% and especially preferably at least 75% or more homology to one another usually remain hybridized to one another. These stringent conditions are known to the skilled worker and described, for example, in Current Protocols in Molecular Biology, John Wiley & Sons, N. Y. (1989), 6.3.1-6.3.6. A preferred nonlimiting example of stringent hybridization conditions is hybridizations in 6 x sodium chloride/sodium citrate (= SSC) at approximately 45°C, followed by one or more washing steps in 0.2 x SSC, 0.1% SDS at 50 to 65°C. The skilled worker knows that these hybridization conditions differ depending on the type of nucleic acid and, for example when organic solvents are present, regarding temperature and buffer concentration. Under "standard hybridization conditions", for example, the hybridization temperature is, depending on the type of nucleic acid, between 42°C and 58°C in aqueous buffer with a concentration of 0.1 to 5 x SSC (pH 7.2). If organic solvent, for example 50% formamide, is present in the abovementioned buffer, the temperature under standard conditions is approximately 42°C. The hybridization conditions for DNA:DNA hybrids, for example, are 0.1 x SSC and 20°C to 45°C, preferably 30°C to 45°C. The hybridization conditions for DNA:RNA hybrids are, for example, 0.1 x SSC and 30°C to 55°C, preferably 45°C to 55°C. The abovementioned hybridization temperatures are determined by way of example for a nucleic acid with approximately 100 bp (= base pairs) in length and with a G + C content of 50% in the absence of formamide. The skilled worker knows how to determine the required hybridization conditions on the basis of the abovementioned textbooks or textbooks such as Sambrook et al., "Molecular Cloning", Cold Spring Harbor Laboratory, 1989; Hames and Higgins (Ed.) 1985, "Nucleic Acids Hybridization: A Practical Approach", IRL Press at Oxford University Press, Oxford; Brown (Ed.) 1991, "Essential Molecular Biology: A Practical Approach", IRL Press at Oxford University Press, Oxford.

In order to determine the percentage of homology (= identity) of two amino acid sequences (for example one of the sequences of SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6 or SEQ ID NO: 8) or of two nucleic acids (for example SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5 or SEQ ID NO: 7), the sequences are written one under the other for an optimal comparison (for example, gaps may be introduced into the sequence of a protein or of a nucleic acid in order to generate an optimal alignment with the other

protein or the other nucleic acid). Then, the amino acid residues or nucleotides at the corresponding amino acid positions or nucleotide positions are compared. If a position in a sequence is occupied by the same amino acid residue or the same nucleotide as the corresponding position in the other sequence, then the molecules are homologous at this position (i.e. amino acid or nucleic acid "homology" as used in the present context corresponds to amino acid or nucleic acid "identity"). The percentage of homology between the two sequences is a function of the number of positions which the sequences share (i.e. % homology = number of identical positions/total number of positions x 100). The terms homology and identity are therefore to be considered as synonymous.

An isolated nucleic acid molecule which encodes an acyl-CoA:lysophospholipid acyltransferase which is homologous to a protein sequence of SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6 or SEQ ID NO: 8 can be generated by introducing one or more nucleotide substitutions, additions or deletions in/into a nucleotide sequence of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5 or SEQ ID NO: 7 so that one or more amino acid substitutions, additions or deletions are introduced in/into the protein which is encoded. Mutations in one of the sequences of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5 or SEQ ID NO: 7 can be introduced by standard techniques such as site-specific mutagenesis and PCR-mediated mutagenesis. It is preferred to generate conservative amino acid substitutions in one or more of the predicted nonessential amino acid residues. In a "conservative amino acid substitution", the amino acid residue is replaced by an amino acid residue with a similar side chain. Families of amino acid residues with similar side chains have been defined in the art. These families comprise amino acids with basic side chains (for example lysine, arginine, histidine), acidic side chains (for example aspartic acid, glutamic acid), uncharged polar side chains (for example glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), unpolar side chains (for example alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (for example threonine, valine, isoleucine) and aromatic side chains (for example tyrosine, phenylalanine, tryptophan, histidine). A predicted nonessential amino acid residue in an acyl-CoA:lysophospholipid acyltransferase is thus preferably replaced by another amino acid residue from the same family of side chains. In another embodiment, the mutations can, alternatively, be introduced randomly over all or part of the sequence encoding the acyl-CoA:lysophospholipid acyltransferase, for example by saturation mutagenesis, and the resulting mutants can be screened by the herein-described acyl-CoA:lysophospholipid acyltransferase activity in order to identify mutants which have retained the acyl-CoA:lysophospholipid acyltransferase activity. Following the mutagenesis of one of the sequences of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5 or SEQ ID NO: 7, the protein which is encoded can be expressed recombinantly, and the activity of the protein can be determined, for example using the tests described in the present text.

The present invention is illustrated in greater detail by the examples which follow, which are not to be construed as limiting. The content of all of the references, patent applications, patents and published patent applications cited in the present patent

application is herewith incorporated by reference.

Examples

Example 1: General methods

a) General cloning methods:

Cloning methods such as, for example, restriction cleavages, agarose gel electrophoresis, purification of DNA fragments, transfer of nucleic acids to nitrocellulose and nylon membranes, linking of DNA fragments, transformation of Escherichia coli and yeast cells, cultivation of bacteria and sequence analysis of recombinant DNA were carried out as described in Sambrook et al. (1989) (Cold Spring Harbor Laboratory Press: ISBN 0-87969-309-6) or Kaiser, Michaelis and Mitchell (1994) "Methods in Yeast Genetics" (Cold Spring Harbor Laboratory Press: ISBN 0-87969-451-3).

b) Chemicals

Unless stated otherwise in the text, the chemicals used were obtained in analytical-grade quality from Fluka (Neu-Ulm, Germany), Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany), Serva (Heidelberg, Germany) and Sigma (Deisenhofen, Germany). Solutions were prepared using purified, pyrogen-free water, referred to as H_2O hereinbelow, from a Milli-Q Water System water purification system (Millipore, Eschborn, Germany). Restriction endonucleases, DNA-modifying enzymes and molecular-biological kits were obtained from AGS (Heidelberg, Germany), Amersham (Brunswick, Germany), Biometra (Göttingen, Germany), Boehringer (Mannheim, Germany), Genomed (Bad Oeynhausen, Germany), New England Biolabs (Schwalbach/Taunus, Germany), Novagen (Madison, Wisconsin, USA), Perkin-Elmer (Weiterstadt, Germany), Pharmacia (Freiburg, Germany), Qiagen (Hilden, Germany) and Stratagene (Amsterdam, the Netherlands). Unless stated otherwise, they were used according to the manufacturer's instructions.

c) Cloning and expression of desaturases and elongases

The Escherichia coli strain XL1 Blue MRF' kan (Stratagene) was used for subcloning Δ6-desaturase from Physcomitrella patens. This gene was functionally expressed using the Saccharomyces cerevisiae strain INVSc 1 (Invitrogen Co.). E. coli was cultured in Luria-Bertani broth (LB, Duchefa, Haarlem, the Netherlands) at 37°C. If necessary, ampicillin (100 mg/liter) was added and 1.5% (w/v) agar was added for solid LB media. S. cerevisiae was cultured at 30°C either in YPG medium or in complete minimal medium without uracil (CMdum; see in: Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K., Albright, L.B., Coen, D.M., and Varki, A. (1995) Current Protocols in Molecular Biology, John Wiley & Sons, New York) with either 2% (w/v) raffinose or glucose. For solid media, 2% (w/v) Bacto™-Agar (Difco) were added. The plasmids used for cloning and expression are pUC18 (Pharmacia) and pYES2 (Invitrogen Co.).

d) Cloning and expression of PUFA-specific desaturases and elongases

For expression in plants, cDNA clones of SEQ ID NO: 9, 11 or 13 were modified so as for only the coding region to be amplified by means of polymerase chain reaction with the aid of two oligonucleotides. Care was taken here to observe a consensus sequence upstream of the start codon, for efficient translation. To this end, either the ATA or the AAA base sequence was chosen and inserted into the sequence upstream of the ATG [Kozak, M. (1986) Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes, Cell 44, 283-2929]. In addition, a restriction cleavage site was introduced upstream of this consensus triplet, which must be compatible with the cleavage site of the target vector into which the fragment is to be cloned and with the aid of which gene expression is to be carried out in micro-organisms or plants.

The PCR reaction was carried out in a thermocycler (Biometra), using plasmid DNA as template and Pfu DNA polymerase (Stratagene) and the following temperature program: 3 min at 96°C, followed by 30 cycles of 30 s at 96°C, 30 s at 55°C and 2 min at 72°C, 1 cycle of 10 min at 72°C and stop at 4°C. The annealing temperature was varied depending on the oligonucleotides chosen. A synthesis time of about one minute per kilobase pair of DNA has to be taken as starting point. Other parameters which influence the PCR, such as, for example, Mg ions, salt, DNA polymerase etc., are familiar to the skilled worker in the field and may be varied as required.

The correct size of the amplified DNA fragment was confirmed by means of agarose-TBE gel electrophoresis. The amplified DNA was extracted from the gel using the QIAquick gel extraction kit (QIAGEN) and ligated into the SmaI restriction site of the dephosphorylated pUC18 vector, using the Sure Clone Ligations Kit (Pharmacia), resulting in the pUC derivatives. After transformation of E. coli XL1 Blue MRF' kan a DNA minipreparation [Riggs, M.G., & McLachlan, A. (1986) A simplified screening procedure for large numbers of plasmid mini-preparation. BioTechniques 4, 310-313] of ampicillin-resistant transformants was carried out, and positive clones were identified by means of BamHI restriction analysis. The sequence of the cloned PCR product was confirmed by means of resequencing using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, Weiterstadt, Germany).

e) Transformation of Agrobacterium

Unless described otherwise, Agrobacterium-mediated plant transformation was carried out with the aid of an Agrobacterium tumefaciens strain, as by Deblaere et al. (1984, Nucl. Acids Res. 13, 4777-4788).

f) Plant transformation

Unless described otherwise, Agrobacterium-mediated plant transformation was carried out using standard transformation and regeneration techniques (Gelvin, Stanton B., Schilperoort, Robert A., Plant Molecular Biology Manual, 2nd ed., Dordrecht: Kluwer

Academic Publ., 1995, in Sect., Ringbuc Zentrale Signatur: BT11-P ISBN 0-7923-2731-4; Glick, Bernard R., Thompson, John E., Methods in Plant Molecular Biology and Biotechnology, Boca Raton: CRC Press, 1993, 360 S., ISBN 0-8493-5164-2).

According thereto, it is possible to transform, for example, oilseed rape by means of cotyledon or hypocotyl transformation (Moloney et al., Plant Cell 8 (1989) 238-242; De Block et al., Plant Physiol. 91 (1989) 694-701). The use of antibiotics for the selection of agrobacteria and plants depends on the binary vector used for transformation and the Agrobacterium strain. Normally, oilseed rape is selected using kanamycin as selectable plant marker.

The transformation of soya may be carried out using, for example, a technique described in EP-A-0 0424 047 (Pioneer Hi-Bred International) or in EP-A-0 0397 687, US 5,376,543, US 5,169,770 (University Toledo).

The transformation of plants using particle bombardment, polyethylene glycol-mediated DNA uptake or via the silicon carbonate fiber technique is described, for example, by Freeling and Walbot "The maize handbook" (1993) ISBN 3-540-97826-7, Springer Verlag New York).

Unless described otherwise, Agrobacterium-mediated gene transfer into linseed (Linum usitatissimum) was carried out by the technique as described in Mlynarova et al. [(1994) Plant Cell Report 13:282-285].

g) Plasmids for plant transformation

Binary vectors based on the vectors pBinAR (Höfgen and Willmitzer, Plant Science 66 (1990) 221-230) or pGPTV (Becker et al. 1992, Plant Mol. Biol. 20:1195-1197) were used for plant transformation. The binary vectors which comprise the nucleic acids to be expressed are constructed by ligating the cDNA in sense orientation into the T-DNA. 5' of the cDNA, a plant promoter activates cDNA transcription. A polyadenylation sequence is located 3' of the cDNA. The binary vectors may carry different marker genes such as, for example, the acetolactate synthase gene (AHAS or ALS) [Ott et al., J. Mol. Biol. 1996, 263:359-360] which imparts a resistance to the imidazolinones or the nptII marker gene which codes for a kanamycin resistance imparted by neomycin phosphotransferase.

Tissue-specific expression of the nucleic acids can be achieved using a tissue-specific promoter. Unless described otherwise, the LeB4 or the USP promoter or the phaseolin promoter was cloned 5' of the cDNA. Terminators used were the NOS terminator and the OCS terminator (see figure 8). Figure 8 depicts a vector map of the vector used for expression, pSUN3CeLPLAT.

It is also possible to use any other seed-specific promoter element such as, for example, the napin or arcelin promoter (Goossens et al. 1999, Plant Phys. 120(4):1095-1103 and Gerhardt et al. 2000, Biochimica et Biophysica Acta 1490(1-

2):87-98).

The CaMV-35S promoter or a v-ATPase C1 promoter can be used for constitutive expression in the whole plant.

The nucleic acids used in the process which encode acyl-CoA:lysophospholipid acyltransferases; desaturases or elongases were cloned into a binary vector one after the other by constructing a plurality of expression cassettes, in order to mimic the metabolic pathway in plants.

Within an expression cassette, the protein to be expressed may be guided into a cellular compartment by using a signal peptide, for example for plastids, mitochondria or the endoplasmic reticulum (Kermode, Crit. Rev. Plant Sci. 15, 4 (1996) 285-423). The signal peptide is cloned 5' of and in frame with the cDNA in order to achieve the subcellular localization of the fusion protein.

Examples of multiexpression cassettes were disclosed in DE 102 19 203 and are given again below.

i.) Promoter-terminator cassettes

Expression cassettes consist of at least two functional units such as a promoter and a terminator. Further desired gene sequences such as targeting sequences, coding regions of genes or parts thereof etc. may be inserted between promoter and terminator. To construct the expression cassettes, promoters and terminators (USP promoter: Baeumlein et al., Mol Gen Genet, 1991, 225 (3):459-67); OCS terminator: Gielen et al. EMBO J. 3 (1984) 835ff.) were isolated with the aid of the polymerase chain reaction and tailor-made with flanking sequences of choice on the basis of synthetic oligonucleotides.

Examples of oligonucleotides which may be used are the following:

USP1 upstream:
- CCGGAATTCGGCGCGCCGAGCTCCTCGAGCAAATTTACACATTGCCA -

USP2 upstream:
- CCGGAATTCGGCGCGCCGAGCTCCTCGAGCAAATTTACACATTGCCA -

USP3 upstream:
- CCGGAATTCGGCGCGCCGAGCTCCTCGAGCAAATTTACACATTGCCA -

USP1 downstream:
- AAAACTGCAGGCGGCCGCCCACCGCGGTGGGCTGGCTATGAAGAAATT -

USP2 downstream:
- CGCGGATCCGCTGGCTATGAAGAAATT -

USP3 downstream:
- TCCCCCGGGATCGATGCCGGCAGATCTGCTGGCTATGAAGAAATT -

OCS1 upstream:
- AAAACTGCAGTCTAGAAGGCCTCCTGCTTTAATGAGATAT -

OCS2 upstream:
- CGCGGATCCGATATCGGGCCCGCTAGCGTTAACCCTGCTTTAATGAGATAT -

OCS3 upstream:
- TCCCCCGGGCCATGGCCTGCTTTAATGAGATAT -

OCS1 downstream:
- CCCAAGCTTGGCGCGCCGAGCTCGAATTCGTCGACGGACAATCAGTAAATTGA -

OCS2 downstream:
- CCCAAGCTTGGCGCGCCGAGCTCGAATTCGTCGACGGACAATCAGTAAATTGA -

OCS3 downstream:
- CCCAAGCTTGGCGCGCCGAGCTCGTCGACGGACAATCAGTAAATTGA -

The methods are known to the skilled worker in the field and are well known from the literature.

In a first step, a promoter and a terminator were amplified via PCR. The terminator was then cloned into a recipient plasmid and, in a second step, the promoter was inserted upstream of the terminator. As a result, an expression cassette was cloned into the basic plasmid. The plasmids pUT1, 2 and 3 were thus generated on the basis of the pUC19 plasmid.

The corresponding constructs or plasmids are defined in SEQ ID NO: 15, 16 to 17. They comprise the USP promoter and the OCS terminator. Based on these plasmids, the construct pUT12 was generated by cutting pUT1 by means of SalI/ScaI and pUT2 by means of XhoI/ScaI. The fragments comprising the expression cassettes were ligated and transformed into E. coli XL1 blue MRF. After isolating ampicillin-resistant colonies, DNA was prepared and those clones which comprise two expression cassettes were identified by restriction analysis. The XhoI/SalI ligation of compatible ends has eliminated here the two cleavage sites, XhoI and SalI, between the expression cassettes. The resulting plasmid, pUT12, is indicated in SEQ ID NO: 18. Subsequently, pUT12 was cut again by means of SalI/ScaI and pUT3 was cut by means of XhoI/ScaI. The fragments comprising the expression cassettes were ligated and transformed into E. coli XLI blue MRF. After isolation from ampicillin-resistant colonies, DNA was again prepared, and those clones which comprise three expression cassettes were identified by restriction analysis. In this manner, a set of multiexpression cassettes was produced which can be utilized for insertion of desired DNA and which is described in table 1 and which moreover can incorporate further

expression cassettes.

Said cassettes comprise the following elements:

Table 1

PUC19 derivative	Cleavage sites upstream of the USP promoter	Multiple cloning cleavage sites	Cleavage sites downstream of the OCS terminator
PUT1	EcoRI/AscI/ SacI/XhoI	BstXI/NotI/ PstI/XbaI/StuI	SalI/EcoRI/ SacI/AscI/ HindIII
PUT2	EcoRI/AscI/ SacI/XhoI	BamHI/EcoRV/ ApaI/NheI/ HpaI	SalI/EcoRI/ SacI/AscI/ HindIII
PUT3	EcoRI/AscI/ SacI/XhoI	BglII/NaeI/ ClaI/SmaI/NcoI	SalI/SacI/ AscI/HindIII
PUT12 double expression cassette	EcoRI/AscI/ SacI/XhoI	BstXI/NotI/ PstI/XbaI/StuI and BamHI/EcoRV/ ApaI/NheI/ HpaI	SalI/EcoRI/ SacI/AscI/ HindIII
PUT123 triple expression cassette	EcoRI/AscI/ SacI/XhoI	1. BstXI/NotI/ PstI/XbaI/StuI and 2. BamHI/EcoRV/ ApaI/NheI/ HpaI and 3. BglII/NaeI/ ClaI/SmaI/NcoI	SalI/SacI/AscI/HindIII

Furthermore, further multiexpression cassettes may be generated, as described and as specified in more detail in table 2, with the aid of the

i) USP promoter or with the aid of the

ii) 700 base pair 3' fragment of the LeB4 promoter or with the aid of the

iii) DC3 promoter and employed for seed-specific gene expression.

The DC3 promoter is described in Thomas, Plant Cell 1996, 263:359-368 and consists merely of the region from -117 to +26, which is why it therefore constitutes one of the smallest known seed-specific promoters. The expression cassettes may comprise several copies of the same promoter or else be constructed via three different promoters.

Advantageously used polylinker- or polylinker-terminator-polylinkers can be found in the sequences SEQ ID NO: 23 to 25.

Table 2: Multiple expression cassettes

Plasmid name of the pUC19 derivative	Cleavage sites upstream of the particular promoter	Multiple cloning cleavage sites	Cleavage sites downstream of the OCS terminator
pUT1 (pUC19 with USP-OCS1)	EcoRI/AscI/SacI/XhoI	(1) BstXI/NotI/PstI/ XbaI/StuI	SalI/EcoRI/SacI/AscI/ HindIII
PDCT (pUC19 with DC3-OCS)	EcoRI/AscI/SacI/XhoI	(2) BamHI/EcoRV/ ApaI/NheI/ HpaI	SalI/EcoRI/SacI/AscI/ HindIII
PleBT (pUC19 with LeB4(700)-OCS)	EcoRI/AscI/SacI/XhoI	(3) BglII/NaeI/ ClaI/SmaI/NcoI	SalI/SacI/AscI/HindIII
PUD12 (pUC 19 with USP-OCS1 and with DC3-OCS)	EcoRI/AscI/SacI/XhoI	(1) BstXI/NotI/ PstI/XbaI/StuI and (2) BamHI/EcoRV/ ApaI/NheI/ HpaI	SalI/EcoRI/SacI/AscI/ HindIII
PUDL123 Triple expression cassette (pUC19 with USP/DC3 and LeB4-700)	EcoRI/AscI/SacI/XhoI	(1) BstXI/NotI/ PstI/XbaI/StuI and (2) BamHI/ (EcoRV*)/ApaI/NheI/ HpaI and (3) BglII/NaeI/ ClaI/SmaI/NcoI	SalI/SacI/AscI/HindIII

* EcoRV cleavage site cuts in the 700 base pair fragment of the LeB4 promoter (LeB4-700)

Further promoters for multigene constructs can be generated analogously, in particular by using the

a) 2.7 kB fragment of the LeB4 promoter or with the aid of the

b) phaseolin promoter or with the aid of the

c) constitutive v-ATPase c1 promoter.

It may be particularly desirable to use further particularly suitable promoters for constructing seed-specific multiexpression cassettes, such as, for example, the napin promoter or the arcelin-5 promoter.

Further vectors which can be utilized in plants and which have one or two or three promoter-terminator expression cassettes can be found in the sequences SEQ ID NO: 26 to SEQ ID NO: 31.

ii.) Generation of expression constructs which comprise promoter, terminator and desired gene sequence for the expression of PUFA genes in plant expression

cassettes.

The Δ6-elongase Pp_PSE1 is first inserted into the first cassette in pUT123 via BstXI and XbaI. Then, the moss Δ6-desaturase (Pp_des6) is inserted via BamHI/NaeI into the second cassette and, finally, the Phaeodactylum Δ5-desaturase (Pt_des5) is inserted via BglII/NcoI into the third cassette (see SEQ ID NO: 19). The triple construct is named pARA1. Taking into consideration sequence-specific restriction cleavage sites, further expression cassettes, as set out in table 3 and referred to as pARA2, pARA3 and pARA4, may be generated.

Table 3: Combinations of desaturases and elongases

Gene plasmid	Δ6-Desaturase	Δ5-Desaturase	Δ6-Elongase
pARA1	Pp_des6	Pt_des5	Pp_PSE1
pARA2	Pt_des6	Pt_des5	Pp_PSE1
pARA3	Pt_des6	Ce_des5	Pp_PSE1
PARA4	Ce_des6	Ce_des5	Ce_PSE1

des5 = PUFA-specific Δ5-desaturase
des6 = PUFA-specific Δ6-desaturase
PSE = PUFA-specific Δ6-elongase
Pt_des5 = Δ5-desaturase from Phaeodactylum tricornutum
Pp_des6 or Pt_des6 = Δ6-desaturase from Physcomitrella patens or Phaeodactylum tricornutum
Pp = Physcomitrella patens, Pt = Phaeodactylum tricornutum
Pp_PSE1 = Δ6-elongase from Physcomitrella patens
Pt_PSE1 = Δ6-elongase from Phaeodactylum tricornutum
Ce_des5 = Δ5-desaturase from Caenorhabditis elegans (Genbank Acc. No. AF078796)
Ce_des6 = Δ6-desaturase from Caenorhabditis elegans (Genbank Acc. No. AF031477, bases 11-1342)
Ce_PSE1 = Δ6-elongase from Caenorhabditis elegans (Genbank Acc. No. AF244356, bases 1-867)

Further desaturases or elongase gene sequences may also be inserted into the expression cassettes of the type described, such as, for example, Genbank Acc. No. AF231981, NM_013402, AF206662, AF268031, AF226273, AF110510 or AF110509.

iii.) Transfer of expression cassettes into vectors for the transformation of Agrobacterium tumefaciens and for the transformation of plants

The constructs thus generated were inserted into the binary vector pGPTV by means of AscI. For this purpose, the multiple cloning sequence was extended by an AscI cleavage site. For this purpose, the polylinker was synthesized de novo in the form of two double-stranded oligonucleotides, with an additional AscI DNA sequence being inserted. The oligonucleotide was inserted into the pGPTV vector by means of EcoRI and HindIII. The cloning techniques required are known to the skilled worker and may readily be found in the literature as described in example 1.

The nucleic acid sequences for Δ5-desaturase (SEQ ID NO: 13), Δ6-desaturase (SEQ ID NO: 9) and Δ6-elongase (SEQ ID NO: 11), which were used for the experiments described below, were the sequences from Physcomitrella patens and Phaeodactylum tricornutum. The corresponding amino acid sequences are the sequences SEQ ID NO: 10, SEQ ID NO: 12 and SEQ ID NO: 14. A vector which comprises all of the

abovementioned genes is indicated in SEQ ID NO: 19. The corresponding amino acid sequences of the genes can be found in SEQ ID NO: 20, SEQ ID NO: 21 and SEQ ID NO: 22.

Example 2: Cloning and characterization of the ceLPLATs

a) Database search

The ceLPLATs (= acyl-CoA:lysophospholipid acyltransferase from Caenorhabditis elegans) were identified by sequence comparisons with known LPA-ATs. The search was restricted to the nematode genome (*Caenorhabditis elegans*) with the aid of the BLAST-Psi algorithm (Altschul et al., J. Mol. Biol. 1990, 215: 403-410), since this organism synthesizes LCPUFAs. The probe employed in the sequence comparison was an LPAAT protein sequence from *Mus musculus* (MsLPAAT Accession No. NP_061350). LPLAT catalyzes, by a reversible transferase reaction, the ATP-independent synthesis of acyl-CoAs from phospholipids with the aid of CoA as cofactor (Yamashita et al., J. Biol. Chem. 2001, 20: 26745-26752). Sequence comparisons enabled two putative ceLPLAT sequences to be identified (Accession No. *T06E8.1* and *F59F4.4*). The identified sequences are most similar to each other and to MsLPAATs (figure 1). The alignment was generated using the Clustal program.

b) Cloning of the CeLPLATs

Primer pairs were synthesized on the basis of the ceLPLAT nucleic acid sequences (table 1) and the corresponding cDNAs were isolated from a *C. elegans* cDNA library by means of PCR processes. The respective primer pairs were selected so as to carry, apart from the start codon, the yeast consensus sequence for high-efficiency translation (Kozak, Cell 1986, 44:283-292). The LPLAT cDNAs were amplified in each case using 2 µl of cDNA-library solution as template, 200 µM dNTPs, 2.5 U of "proof-reading" *pfu* polymerase and 50 pmol of each primer in a total volume of 50 µl. The conditions for the PCR were as follows: first denaturation at 95°C for 5 minutes, followed by 30 cycles at 94°C for 30 seconds, 58°C for one minute and 72°C for 2 minutes, and a final extension step at 72°C for 10 minutes. The sequence of the LPLAT cDNAs was confirmed by DNA sequencing.

Table 4: Nucleotide sequences of the PCR primers for cloning CeLPLATs

Primer	Nucleotide sequence
5' T06E8.1f*	5' ACATAATGGAGAACTTCTGGTCGATCGTC 3'
3' T06E8.1r*	5' TTACTCAGATTTCTTCCCGTCTTT 3'

5' F59F4.4f*	5' ACATAATGACCTTCCTAGCCATATTA 3'
3' F59F4.4r*	5' TCAGATATTCAAATTGGCGGCTTC 3'

* f: forward, r: reverse

Example 3: Analysis of the effect of the recombinant proteins on production of the desired product

a) Possible preparation methods

The effect of genetic modification in fungi, algae, ciliates or, as described in the examples hereinabove, on the production of the polyunsaturated fatty acids in yeasts, or in plants may be determined by growing the modified microorganisms or the modified plant under suitable conditions (such as those described above) and studying the medium and/or the cellular components for increased production of the lipids or fatty acids. These analytical techniques are known to the skilled worker and comprise spectroscopy, thin layer chromatography, various types of staining methods, enzymic and microbiological methods and analytical chromatography such as high-performance liquid chromatography (see, for example, Ullmann, Encyclopedia of Industrial Chemistry, vol. A2, pp. 89-90 and pp. 443-613, VCH: Weinheim (1985); Fallon, A., et al., (1987) "Applications of HPLC in Biochemistry" in: Laboratory Techniques in Biochemistry and Molecular Biology, vol. 17; Rehm et al. (1993) Biotechnology, vol. 3, chapter III: "Product recovery and purification", pp. 469-714, VCH: Weinheim; Belter, P.A., et al. (1988) Bioseparations: downstream processing for Biotechnology, John Wiley and Sons; Kennedy, J.F., and Cabral, J.M.S. (1992) Recovery processes for biological Materials, John Wiley and Sons; Shaeiwitz, J.A., and Henry, J.D. (1988) Biochemical Separations, in: Ullmann's Encyclopedia of Industrial Chemistry, vol. B3; chapter 11, pp. 1-27, VCH: Weinheim; and Dechow, F.J. (1989) Separation and purification techniques in biotechnology, Noyes Publications).

Apart from the abovementioned methods for detecting fatty acids in yeasts, plant lipids are extracted from plant material as described by Cahoon et al. (1999) Proc. Natl. Acad. Sci. USA 96 (22):12935-12940, and Browse et al. (1986) Analytic Biochemistry 152:141-145. The qualitative and quantitative analysis of lipids or fatty acids is described in Christie, William W., Advances in Lipid Methodology, Ayr/Scotland: Oily Press (Oily Press Lipid Library; 2); Christie, William W., Gas Chromatography and Lipids. A Practical Guide - Ayr, Scotland: Oily Press, 1989, Repr. 1992, IX, 307 S. (Oily Press Lipid Library; 1); "Progress in Lipid Research, Oxford: Pergamon Press, 1 (1952) 16 (1977) under the title.: Progress in the Chemistry of Fats and Other Lipids CODEN.

Thus, fatty acids or triacylglycerol (= TAG, abbreviations indicated in brackets) may be analyzed, for example, by means of fatty acid methyl esters (= FAME), gas liquid

chromatography-mass spectrometry (= GC-MS) or thin layer chromatography (TLC).

Unequivocal proof for the presence of fatty acid products may be obtained by means of analyzing recombinant organisms following standard analytical procedures: GC, GC-MS or TLC, as variously described by Christie and references therein (1997, in: Advances on Lipid Methodology, fourth ed.: Christie, Oily Press, Dundee, 119-169; 1998, Gaschromatographie-Massenspektrometrie-Verfahren [Gas chromatography-mass spectrometry methods], Lipide 33:343-353).

The plant material to be analyzed may for this purpose be disrupted either by sonification, glass milling, liquid nitrogen and grinding or via other applicable processes. After the material has been disrupted, it is then centrifuged. The sediment is then resuspended in distilled water, heated at 100°C for 10 min, cooled on ice and centrifuged again, followed by extraction in 0.5 M sulfuric acid in methanol containing 2% dimethoxypropane for 1 h at 90°C, leading to hydrolyzed oil and lipid compounds which result in transmethylated lipids. These fatty acid methyl esters may then be extracted in petroleum ether and finally be subjected to GC analysis using a capillary column (Chrompack, WCOT Fused Silica, CP-Wax-52 CB, 25 µm, 0.32 mm), with a temperature gradient of between 170°C and 240°C for 20 min and at 240°C for 5 min. The identity of the resulting fatty acid methyl esters can be defined using standards available from commercial sources (i.e. Sigma).

In the case of fatty acids for which no standards are available, the identity may be shown via derivatization and subsequent GC-MS analysis. For example, the localization of triple-bond fatty acids is shown via GC-MS after derivatization with 4,4-dimethoxyoxazoline derivatives (Christie, 1998, see above).

b) Fatty acid analysis in plants

Total fatty acids were extracted from plant seeds and analyzed by means of gas chromatography.

The seeds were taken up with 1% sodium methoxide in methanol and incubated at RT (approx. 22°C) for 20 min. This was followed by washing with NaCl solution and taking up the FAMEs in 0.3 ml of heptane.

The samples were fractionated on a ZEBRON-ZB Wax capillary column (30 m, 0.32 mm, 0.25 µm; Phenomenex) in a Hewlett Packard 6850 gas chromatograph with flame ionization detector. The oven temperature was programmed from 70°C (hold for 1 min) to 200°C at a rate of 20°C/min, then to 250°C (hold for 5 min) at a rate of 5°C/min and finally to 260°C at a rate of 5°C/min. The carrier gas used was nitrogen (4.5 ml/min at 70°C). The fatty acids were identified by comparison with retention times of FAME standards (SIGMA).

Example 4: Functional characterization of CeLPLATs in yeast

a) Heterologous expression in Saccharomyces cerevisiae

To characterize the function of the *C. elegans* CeLPLATs, the open reading frames of the particular cDNAs were cloned downstream of the galactose-inducible GAL1 promoter of pYes2.1Topo, using the pYes2.1TOPO TA Expression Kit (Invitrogen), resulting in pYes2-T06E8.1 and pYes2-F59F4.4.

Since expression of the CeLPLATs should result in an efficient exchange of the acyl substrates, the double construct pESCLeu-PpD6-Pse1 which includes the open reading frames of a Δ6-desaturase (PpD6) and a Δ6-elongase (PSE1) from *Physcomitrella patens* (see DE 102 19 203) was also prepared. The nucleic acid sequence of said Δ6-desaturase (PpD6) and said Δ6-elongase (Pse1) are indicated in each case in SEQ ID NO: 9 and SEQ ID NO: 11. The corresponding amino acid sequences can be found in SEQ ID NO: 10 and SEQ ID NO: 12.

The *Saccharomyces cerevisiae* strains C13ABYS86 (protease-deficient) and INVSc1 were transformed simultaneously with the vectors pYes2-T06E8.1 and pESCLeu-PpD6-Pse1 and, respectively, pYes2-F59F4.4 and pESCLeu-PpD6-Pse1 by means of a modified PEG/lithium acetate protocol. The control used was a yeast which was transformed with the pESCLeu-PpD6-Pse1 vector and the empty vector pYes2. The transformed yeasts were selected on complete minimal medium (CMdum) agar plates containing 2% glucose but no uracil or leucine. After selection, 4 transformants, two pYes2-T06E8.1/pESCLeu-PpD6-Pse1 and two pYes2-F59F4.4/pESCLeu-PpD6-Pse1 and one pESCLeu-PpD6-Pse1/pYes2 were selected for further functional expression. The experiments described were also carried out in the yeast strain INVSc1.

In order to express the CeLPLATs, precultures of in each case 2 ml of CMdum liquid medium containing 2% (w/v) raffinose but no uracil or leucine were first inoculated with the selected transformants and incubated at 30°C, 200 rpm, for 2 days. 5 ml of CMdum liquid medium (without uracil and leucine) containing 2% raffinose, 1% (v/v) Tergitol NP-40 and 250 µM linoleic acid ($18:2^{\Delta 9,12}$) or linolenic acid ($18:3^{\Delta 9,12,15}$) were then inoculated with the precultures to an OD_{600} of 0.08. Expression was induced at an OD_{600} of 0.2-0.4 by adding 2% (w/v) galactose. The cultures were incubated at 20°C for a further 48 h.

Fatty acid analysis

The yeast cells from the main cultures were harvested by centrifugation (100 x g, 10 min, 20°C) and washed with 100 mM $NaHCO_3$, pH 8.0 in order to remove residual medium and fatty acids. Fatty acid methyl esters (FAMEs) were prepared from the yeast cell sediments by acidic methanolysis. For this, the cell sediments were incubated with 2 ml of 1N methanolic sulfuric acid and 2% (v/v) dimethoxypropane at 80°C for 1 h. Extraction of the FAMES was carried out by extracting twice with petroleum ether (PE). Nonderivatized fatty acids were removed by washing the organic phases in each case once with 2 ml of 100 mM $NaHCO_3$, pH 8.0 and 2 ml of distilled water. The PE phases were subsequently dried with Na_2SO_4, evaporated under argon

and taken up in 100 µl of PE. The samples were separated on a DB-23 capillary column (30 m, 0.25 mm, 0.25 µm, Agilent) in a Hewlett-Packard 6850 gas chromatograph with flame ionization detector. The conditions for the GLC analysis were as follows: the oven temperature was programmed from 50°C to 250°C at a rate of 5°C/min and finally at 250°C (hold) for 10 min.

The signals were identified by comparing the retention times with those of corresponding fatty acid standards (Sigma).

Acyl-CoA analysis

The acyl-CoA analysis was carried out as described in Larson and Graham (2001; Plant Journal 25: 115-125).

Expression analysis

Figures 2 A and B and figures 3 A and B depict the fatty acid profiles of transgenic C13ABYS86 yeasts fed with $18:2^{\Delta 9,12}$ and $18:3^{\Delta 9,12,15}$, respectively. The substrates fed can be detected in large amounts in all transgenic yeasts. All four transgenic yeasts display synthesis of $18:3^{\Delta 6,9,12}$ and $20:3^{\Delta 8,11,14}$ and, respectively, $18:4^{\Delta 6,9,12,15}$ and $20:4^{\Delta 8,11,14,17}$, the products of the Δ6-desaturase and Δ6-elongase reactions, meaning that the genes PpD6 and Pse1 were able to be functionally expressed.

Figure 2 depicts, as described above, the fatty acid profiles of transgenic C13ABYS86 *S. cerevisiae* cells. The fatty acid methyl esters were synthesized by acidic methanolysis of intact cells which had been transformed either with the pESCLeu-PpD6-Pse1/pYes2 (**A**) or with the pYes2-T06E8.1/pESCLeu-PpD6-Pse1 (**B**) vectors. The yeasts were cultured in minimal medium in the presence of $18:2^{\Delta 9,12}$. The fatty acid methyl esters were subsequently analyzed by GLC.

In the control yeasts transformed with the pESCLeu-PpD6-Pse1/pYes2 vectors, the proportion of $20:3^{\Delta 8,11,14}$ to which $18:3^{\Delta 6,9,12}$ is elongated by Pse1 is substantially lower than in the yeasts which additionally express LPLAT T06E8.1. In fact, elongation of $18:3^{\Delta 6,9,12}$ and $18:4^{\Delta 6,9,12,15}$ was improved by 100-150% by additional expression of CeLPLAT (T06E8.1) (figure 4). This significant increase in the LCPUFA content can be explained only as follows: the exogenously fed fatty acids ($18:2^{\Delta 9,12}$ and $18:3^{\Delta 9,12,15}$, respectively) are first incorporated into phospholipids and desaturated there by Δ6-desaturase to give $18:3^{\Delta 6,9,12}$ and $18:4^{\Delta 6,9,12,15}$. Only after reequilibration with the acyl-CoA pool, $18:3^{\Delta 6,9,12}$ and $18:4^{\Delta 6,9,12,15}$ can be elongated by the elongase to give $20:3^{\Delta 8,11,14}$- and $20:4^{\Delta 8,11,14,17}$-CoA, respectively and then incorporated again into the lipids. LPLAT T06E8.1 is capable of converting the Δ6-desaturated acyl groups very efficiently back to CoA thioesters. Interestingly, it was also possible to improve the elongation of the fed fatty acids $18:2^{\Delta 9,12}$ and $18:3^{\Delta 9,12,15}$. (Figures 2 A and B and figures 3 A and B, respectively).

Figure 3 indicates the fatty acid profiles of transgenic C13ABYS86 *S. cerevisiae* cells.

Synthesis of the fatty acid methyl esters was carried out by acidic methanolysis of intact cells which had been transformed either with the vectors pESCLeu-PpD6-Pse1/pYes2 (**A**) or with the vectors pYes2-T06E8.1/pESCLeu-PpD6-Pse1 (**B**). The yeasts were cultured in minimal medium in the presence of $18:3^{\Delta 9,12,15}$. The fatty acid methyl esters were subsequently analyzed via GLC.

In contrast, expression of a different CeLPLAT (F59F4.4) has no influence on elongation (figure 4). F59F4.4 evidently does not encode an LPLAT. Thus, not every putative LPLAT nucleic acid sequence is enzymatically active in the reaction found according to the invention.

Figure 4 indicates the elongation of exogenously applied $18:2^{\Delta 9,12}$ and $18:3^{\Delta 9,12,15}$, following their endogenous Δ6-desaturation (data of figs. 2 and 3). The exogenously fed fatty acids are first incorporated into phospholipids and desaturated there to give $18:3^{\Delta 6,9,12}$ and $18:4^{\Delta 6,9,12,15}$. Only after reequilibration with the acyl-CoA pool can $18:3^{\Delta 6,9,12}$ and $18:4^{\Delta 6,9,12,15}$ be elongated by the elongase to give $20:3^{\Delta 8,11,14}$- and $20:4^{\Delta 8,11,14,17}$-CoA, respectively, and then incorporated again into the lipids. LPLAT T06E8.1 is capable of converting the Δ6-desaturated acyl groups efficiently back to CoA-thioesters.

These results show that CeLPLAT (T06E8.1) after coexpression with Δ6-desaturase and Δ6-elongase, leads to efficient production of C20-PUFAs. These results can be explained by the fact that CeLPLAT (T06E8.1) makes possible an efficient exchange of the newly synthesized fatty acids between lipids and the acyl-CoA pool (see figure 7).

Figure 7 indicates the acyl-CoA composition of transgenic INVSc1 yeasts transformed with the pESCLeu PpD6Pse1/pYes2 (**A**) or pESCLeu-PpD6-Pse1/pYes2-T06E8.1 (**B**) vectors. The yeast cells were cultured in minimal medium without uracil and leucine in the presence of 250 µM $18:2^{\Delta 9,12}$. The acyl-CoA derivatives were analyzed via HPLC.

When using the yeast strain INVSc1 for coexpression of CeLPLAT (T06E8.1) together with PpD6 and Pse1, the following picture emerges: control yeasts expressing PpD6 and Pse1 comprise, as already shown when using the strain C13ABYS86, only small amounts of the elongation product ($20:3^{\Delta 8,11,14}$, with 18:2 feed, and $20:4^{\Delta 8,11,14,17}$, with 18:3 feed; see figures 5 A and 6 A, respectively). Additional expression of CeLPLAT (T06E8.1) results in a marked increase in these elongation products (see figures 5 B and 6 B). Table 6 indicates that additional expression of CeLPLAT surprisingly causes an 8 fold increase in the $20:3^{\Delta 8,11,14}$ (with 18:2 feed) and, respectively, the $20:4^{\Delta 8,11,14,17}$ (with 18:3 feed) content. It is also revealed that $C16:2^{\Delta 6,9}$ is also elongated more efficiently to give $C18:2^{\Delta 6,9}$.

Table 5: Fatty acid composition (in mol%) of transgenic yeasts transformed with the pESCLeu PpD6Pse1/pYes2 (PpD6 Pse1) or pESCLeu-PpD6-Pse1/pYes2-T06E8.1 (PpD6 Pse1 + T06E8) vectors. The yeast cells were cultured in minimal medium without uracil and leucine in the presence of 250 µM 18:2 $^{\Delta 9,12}$ or 18:3$^{\Delta 9,12,15}$. The fatty acid methyl esters were obtained by acidic methanolysis of whole cells and analyzed via GLC. Each value indicates the average (n = 4) ± standard deviation.

Fatty acids	Feeding with 250 µM 18:2$^{\Delta 9,12}$		Feeding with 250 µM 18:3$^{\Delta 9,12,15}$	
	Pp□6/Pse1	Pp□6/Pse1+ T06E8	Pp□6/Pse1	Pp□6/Pse1+ T06E8
16:0	15.31 ± 1.36	15.60 ± 1.36	12.20 ± 0.62	16.25 ± 1.85
16:1$^{\delta 9}$	23.22 ± 2.16	15.80 ± 3.92	17.61 ± 1.05	14.58 ± 1.93
18:0	5.11 ± 0.63	7.98 ± 1.28	5.94 ± 0.71	7.52 ± 0.89
18:1$^{\delta 9}$	15.09 ± 0.59	16.01 ± 2.53	15.62 ± 0.34	15.14 ± 2.61
18:1$^{\delta 11}$	4.64 ± 1.09	11.80 ± 1.12	4.56 ± 0.18	13.07 ± 1.66
18:2$^{\delta 9,12}$	28.72 ± 3.25	14.44 ± 1.61	-	-
18:3$^{\delta 6,9,12}$	3.77 ± 0.41	4.72 ± 0.72	-	-
18:3$^{\delta 9,12,15}$	-	-	32.86 ± 1.20	14.14 ± 2.52
18:4$^{\delta 6,9,12,15}$	-	-	5.16 ± 1.04	3.31 ± 1.15
20:2$^{\delta 11,14}$	2.12 ± 0.86	4.95 ± 4.71	-	-
20:3$^{\delta 8,11,14}$	1.03 ± 0.14	8.23 ± 1.59	-	-
20:3$^{\delta 11,14,17}$	-	-	4.12 ± 1.54	6.95 ± 2.52
20:4$^{\delta 8,11,14,17}$	-	-	1.34 ± 0.28	8.70 ± 1.11

The fatty acid profile of transgenic INVSc1 S. cerevisiae cells can be found in figure 5. The fatty acid methyl esters were synthesized by acidic methanolysis of intact cells which had been transformed either with the pESCLeu-PpD6-Pse1/pYes2 (A) or with the pYes2-T06E8.1/pESCLeu-PpD6-Pse1 (B) vectors. The yeasts were cultured in minimal medium in the presence of 18:2$^{\Delta 9,12}$. The fatty acid methyl esters were subsequently analyzed via GLC.

Figure 6 depicts the fatty acid profiles of transgenic INVSc1 *S. cerevisiae* cells. The fatty acid methyl esters were synthesized by acidic methanolysis of intact cells which had been transformed either with the pESCLeu-PpD6-Pse1/pYes2 (**A**) or with the pYes2-T06E8.1/pESCLeu-PpD6-Pse1 (**B**) vectors. The yeasts were cultured in minimal medium in the presence of $18:3^{\Delta,12,15}$. The fatty acid methyl esters were subsequently analyzed via GLC.

A measure for the efficiency of LCPUFA biosynthesis in transgenic yeast is the quotient of the content of the desired Δ6-elongation product after Δ6-desaturation ($20:3^{\Delta8,11,14}$ and $20:4^{\Delta8,11,14,17}$, respectively) to the content of fatty acid fed in ($18:2^{\Delta9,12}$ and $18:3^{\Delta9,12,15}$, respectively). This quotient is 0.04 in INVSc1 control yeasts expressing PpD6 and Pse1, and 0.60 in yeasts expressing CeLPLAT in addition to PpD6 and Pse1. In other words: the content of desired Δ6-elongation product after Δ6-desaturation with coexpression of CeLPLAT is 60% of the content of the fatty acid fed in in each case. In control yeasts, this content is only approx. 4%, meaning a 15 fold increase in the efficiency of LCPUFA biosynthesis in transgenic yeast due to coexpression of LPLAT.

Interestingly, coexpression of CeLPLAT causes not only an increase in the elongation products mentioned, $20:3^{\Delta8,11,14}$ and $20:4^{\Delta8,11,14,17}$, but also an increase in the $20:3^{\Delta8,11,14}$: $20:2^{\Delta11,14}$ ratio and the $20:4^{\Delta8,11,14,17}$: $20:3^{\Delta11,14,17}$ ratio, respectively. This means that, in the presence of LPLAT, Δ6-elongase preferably uses polyunsaturated fatty acids ($18:3^{\Delta6,9,12}$ and $18:4^{\Delta6,9,12,15}$) as substrate, while no distinct substrate specificity is discernible in the absence of LPLAT ($18:2^{\Delta9,12}$ and $18:3^{\Delta9,12,15}$ are also elongated). The reason for this may be protein-protein interactions between Δ6-elongase, Δ6-desaturase and LPLAT or posttranslational modifications (partial proteolysis, for example). This will also explain why the above-described rise in Δ6-elongation products with coexpression of Δ6-desaturase, Δ6-elongase and LPLAT is smaller when a protease-deficient yeast strain is used.

Acyl-CoA analyses of transgenic INVSc1 yeasts fed with $18:2^{\Delta9,12}$ gave the following result: no $18:3^{\Delta6,9,12}$-CoA and $20:3^{\Delta8,11,14}$-CoA is detectable in control yeasts expressing PpD6 and Pse1, indicating that neither the substrate ($18:3^{\Delta6,9,12}$-CoA) nor the product ($20:3^{\Delta8,11,14}$-CoA) of Δ6-elongase is present in detectable amounts in control yeasts. This suggests that the transfer of $18:3^{\Delta6,9,12}$ from membrane lipids into the acyl-CoA pool does not take place or does not take place correctly, meaning that there is hardly any substrate available for the Δ6-elongase present, and this in turn explains the low elongation product content in control yeasts. INVSc1 yeasts which express CeLPLAT in addition to PpD6 and Pse1 and which had been fed with $18:2^{\Delta9,12}$ have substantial amounts of $20:3^{\Delta8,11,14}$-CoA but not of $18:3^{\Delta6,9,12}$-CoA. This indicates that LPLAT transfers $18:3^{\Delta6,9,12}$ from the membrane lipids to the acyl-CoA pool very efficiently. $18:3^{\Delta6,9,12}$-CoA is then elongated by Δ6-elongase so that $20:3^{\Delta8,11,14}$-CoA but not any $18:3^{\Delta6,9,12}$-CoA is detectable.

b) Functional characterization of the CeLPLATs in transgenic plants

Expression of functional CeLPLAT in transgenic plants

DE 102 19 203 describes transgenic plants whose seed oil comprises small amounts of ARA and EPA, due to seed-specific expression of functional genes coding for Δ6-desaturase, Δ6-elongase and Δ5-desaturase. The vector exploited for transformation of these plants can be found in SEQ ID NO: 19. In order to increase the content of these LCPUFAs, the gene CeLPLAT (T06E8.1) was additionally expressed in seeds in the transgenic plants mentioned.

For this purpose, the coding region of CeLPLAT was amplified via PCR.

Table 6 indicates the primers used for cloning another CeLPLAT clone into binary vectors.

Table 6: Nucleotide sequences of the PCR primers for cloning CeLPLAT (T06E8.1) into the binary vector pSUN3

Primer	Nucleotide sequence
ARe503f*	5' TTAAGCGCGGCCGCATGGAGAACTTCTGGTCG 3'
ARe504r*	5' ACCTCGGCGGCCGCCCTTTTACTCAGATTTC 3'

* f: forward, r: reverse

The PCR product was cloned into a pENTRY vector between USP promoter and OCS terminator. The expression cassette was then cloned into the binary pSUN300 vectors. The vector obtained was referred to as pSUN3CeLPLAT (figure 8). In addition, the CeLPLAT coding regions were amplified and cloned between LegB4 promoter and OCS terminator. This vector was referred to as pGPTVCeLPLAT (figure 9A).

In addition, the CeLPLAT coding regions were amplified via PCR and cloned between LegB4 promoter and OCS terminator. The PCR primers used for this were selected so as for an efficient Kozak sequence to be introduced into the PCR product. Moreover, the CeLPLAT DNA sequence was modified so as to adapt to the codon usage of higher plants.

The following primers were used for the PCR:

Forward primer: 5'-ACATAATGGAGAACTTCTGGTCTATTGTTGTGTTTTTTCTA-3'

Reverse primer: 5'- CTAGCTAGCTTACTCAGATTTCTTCCCGTCTTTTGTTTCTC-3'

The PCR product was cloned into the cloning vector pCR Script and cloned via the restriction enzymes XmaI and SacI into the vector pGPTV LegB4-700. The resulting plasmid was referred to as pGPTV LegB4-700 + T06E8.1 (figure 9A).

The same PCR product was in addition cloned into a multi-gene expression vector which already comprised the genes for a Phaeodactylum tricornutum delta-6-desaturase (SEQ ID NO: 32, amino acid sequence SEQ ID NO: 33) and a P. patens delta-6-elongase. The resulting plasmid was referred to as pGPTV USP/OCS-1,2,3 PSE1(Pp)+D6-Des(Pt)+2AT (T06E8-1) (figure 9B). The sequences of the vector and of the genes can be found in SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36 and SEQ ID NO: 37. The Phaeodactylum tricornutum Δ6-desaturase extends from nucleotide 4554 to 5987 in SEQ ID NO: 34. The Physcomitrella patens Δ6-elongase extends from nucleotide 1026 to 1898 and that of Caenorhabditis elegans LPLAT extends from nucleotide 2805 to 3653 in SEQ ID NO: 34.

Tobacco plants were cotransformed with the pSUN3CeLPLAT vector and the vector described in DE 102 19 203 and SEQ ID NO: 19, which comprises genes coding for Δ6-desaturase, Δ6-elongase and Δ5-desaturase, with transgenic plants being selected using kanamycin.

Tobacco plants were moreover transformed with the pGPTV USP/OCS-1,2,3 PSE1(Pp)+D6-Des(Pt)+2AT (T06E8-1) vector [see SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36 and SEQ ID NO: 37].

Linseed was transformed with the pSUN3CeLPLAT vector. The resulting transgenic plants were crossed with those transgenic linseed plants which already comprised small amounts of ARA and EPA, owing to functional gene expression of Δ6-desaturase, Δ6-elongase and Δ5-desaturase.

Linseed was furthermore transformed with the pGPTV LegB4-700 + T06E8.1 vector. The resulting transgenic plants were crossed with those transgenic linseed plants which already comprised small amounts of ARA and EPA, owing to functional expression of Δ6-desaturase, Δ6-elongase and Δ5-desaturase.

The seeds of transgenic tobacco and linseed plants were, as described hereinbefore [example 3 b)], studied for increased LCPUFAs contents.

The function of acyl-CoA:lysophopholipid acyltransferase (LPLAT) can be deduced from the studies presented herein as depicted in figure 10. The biosynthetic pathway of LCPUFAS is thus as follows.

Desaturases catalyze the introduction of double bonds into lipid-coupled fatty acids (*sn2*-acyl-phosphatidylcholine), while the elongases exclusively catalyze the elongation of coenzyme A-esterified fatty acids (acyl-CoAs). According to this mechanism, the alternating action of desaturases and elongases requires continuous exchange of acyl substrates between phospholipids and acyl-CoA pool and thus the existence of an additional activity which converts the acyl substrates to the substrate form required in each case, i.e. lipids (for desaturases) or CoA thioesters (for elongases). This exchange between acyl-CoA pool and phospholipids is made possible by LCPUFA-specific LPLAT. The biosynthesis of ARA (**A**) takes place analogously to that of EPA (**B**), but with the difference that, in the case of EPA, a Δ15-desaturation takes place upstream of the Δ6-desaturation so that α18:3-PC acts as a substrate for Δ6-desaturase. The biosynthesis of DHA requires a further exchange between phospholipids and acyl-CoA pool via LPLAT: $20:5^{\Delta5,8,11,14,17}$ is transferred from the phospholipids pool to the CoA pool and, after Δ5-elongation, $22:5^{\Delta7,10,13,16,19}$ is transferred from the CoA pool to the phospholipids pool and finally converted by Δ4-desaturase to give DHA. The same applies to the exchange in the biosynthetic pathway using Δ8-desaturase, Δ9-elongase and Δ5-desaturase.

Equivalents

Many equivalents of the specific embodiments of the invention described herein can be identified or found by the skilled worker by using merely routine experiments. These equivalents are intended to be within the scope of the patent claims.